THE DEMONSTRATION OF OESTRUS IN THE VITAMIN A-DEFICIENT RAT BY SUPRAVITAL STUDY OF THE VAGINAL SMEARS [1]

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TWO FIGURES

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In the preceding report (Mason and Ellison, '35), it was observed that regular oestrous cycles could be followed, by dry stained smears, in A-deficient rats until the variations in the daily smears became obliterated by excessive keratinization of the vaginal epithelium. That oestrous changes still occurred was indicated by the successful matings secured when normal males were placed with the deficient females, confirming similar observations made by Evans and Bishop ('22) and by Evans ('28).

Guttmacher ('26) observed that the cellular changes of oestrus could be readily followed in the normal rat by examination of the living cell types in lavages obtained by pipetting physiological saline, containing small amounts of neutral red, into the vagina. He observed that nucleated epithelial cells were sloughed into the vaginal lumen in prooestrus, cornified cells in oestrus, and active leucocytes were found in large numbers in metoestrus. As dioestrus progressed, the leucocytes became fewer in number and showed degenerative changes, indicating that the leucocytic invasion occurred chiefly at metoestrus. This same method has been successfully

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applied to the study of vaginal changes in the A-deficient rat by Aberle ('33).

Application of this technic, some 4 years ago, proved of great assistance in interpreting the cellular changes observed in the dry stained vaginal smears previously described (Mason and Ellison, '35). Its further application has made possible the demonstration of cyclic vaginal changes in the more advanced stages of A-deficiency, where the routine dry smear proved inadequate. The present report will deal with the results of the latter study, together with an analysis of the factors involved in the irregularity of the oestrous changes noted in rats suffering from severe depletion of vitamin A.

EXPERIMENTAL

The dietary and other general experimental procedures were essentially the same as those used in studies reported in the preceding report (Mason and Ellison, '35), and require no further elaboration at this time.

Supravital vaginal smears were made, following the technic of Guttmacher ('26), by pipetting into the vagina 1 cc. of a 1 to 10,000 solution of neutral red, made up in normal saline. The cellular suspension obtained was withdrawn by the pipette and placed on clean cover glasses. The latter were then inverted on specially prepared neutral red slides, as used in blood studies in this laboratory. The cover slips were rimmed with cedar oil and kept in a warm chamber at 37.5°C. while the counts were being made. Two hundred cells were counted and a general survey of the complete smear made in order to compensate for any clumping of cells.

1. Changes in the supravital vaginal smears of A-deficient rats

The cyclic changes in the vaginal smears of A-deficient rats up to the time at which cornification became complete and continuous have been fully described in a separate report (Mason and Ellison, '35). When this latter condition was reached, the supravital staining of the smear became particularly useful. The general appearance of these smears representative of severe A-depletion, will be briefly outlined.

In general, the daily sequence of events was represented by smears containing cornified cells only, for a period of 2 to 4 days, alternating with smears in which variable numbers of leucocytes and a few nucleated epithelial cells appeared intermingled with cornified cells, for a period of 2 to 4 days. The periods of prolonged and complete cornification resembled those observed in dry stained smears (fig. 14, Mason and Ellison, '35) the last day of which represented the period of true oestrus as demonstrated by the invariable acceptance of the male at this time. The periods of leucocytic infiltration were comparable to those observed in dry stained smears (figs. 12 and 13, Mason and Ellison, '35) before cornification became complete and continuous.

The latter type of smear showed certain variations during the interval between periods of complete cornification. During the early phases of this interval the leucocytes were particularly abundant and very active, as indicated by their capacity to take up neutral red. This we interpreted as representing the period of metoestrus. During the middle and latter part of this interval the leucocytes not only decreased in number, but showed much less evidence of activity and more of them appeared to be in a dying state. This stage was considered to represent the dioestrous interval, and was immediately followed by several days of complete cornification, during which no distinction could be made between the stages of prooestrus and of oestrus.

From what has been said above, it is apparent that the supravital method merely permitted more ready detection of the appearance of leucocytes which were intermingled with cornified cells[2] at periods when the process of cornification

[2] The large cornified non-nucleated cells, observed in all stages of the cycle, contained within their cytoplasm large numbers of rod-like particles in active brownian movement. Application of Gram's stain to dried fixed smears of these cells has demonstrated that at least many of these particles were bacteria. Culture media innoculated with these cells have also demonstrated the presence of many organisms, largely of the colon bacillus type, either in the cells themselves or in the vaginal contents. Though such organisms are present in the vagina of normal rats, comparisons with similar cultures from the latter indicated that the bacterial flora was definitely increased in A-deficient rats.

had become so extreme that the leucocytes were not readily demonstrable in the dry stained smears.

2. Variability in length of the oestrous cycle

Slight increases in the length of the oestrous cycle were sometimes observed in A-deficient rats in which increment in growth had ceased. During more advanced stages of A-deficiency, associated with marked xerophthalmia and loss of body weight, it was not unusual to observe cycles lasting from 8 to 20 days. Moreover, rats which failed to attain a weight of more than 100 to 110 gm. on the deficient diet usually exhibited a complete absence of oestrous activity, even though the deficiency symptoms were less severe than in other rats showing fair growth and only slightly irregular cycles. Frequently, this same phenomenon appeared during an unusually rapid growth response following administration of vitamin A to animals in a very depleted state.

Study of the vaginal smears demonstrated that the lengthening of the oestrous cycle was due, almost entirely, to prolongation of the dioestrous interval. The periods of complete cornification rarely exceeded 3 or 4 days in duration. In view of this apparent relationship between the lengthened oestrous cycles and associated growth disturbances of A-deficient rats, it seemed essential to analyze this problem more carefully by means of more carefully controlled conditions.

a. Comparison of A-deficency and inanition. The oestrous cycles of A-deficient female rats were contrasted with those of littermate sisters fed the same diet made adequate by the addition of cod liver oil, but restricted in the amount given such that their growth rate was approximately the same as that of the former group (fig. 1). In order to maintain a moderately severe state of A-deficiency, without endangering the life of the animal, it was necessary to administer small doses of diluted cod liver oil, orally, at various times during the first 120 days of experiment (periods A and B). This amounted to a total of approximately 10 drops of cod liver oil for each of the three A-deficient rats. The latter exhibited

marked vaginal cornification and mild to moderately severe xerophthalamia from the 40th to the 120th day of experiment (period B), their oestrous cycles averaging 14.3 days in length, as compared with an average of 15.3 days for the 'restricted' controls during the same period.

The deficient rats were then given a single dose of three drops of cod liver oil on the 120th day; sufficient to produce a

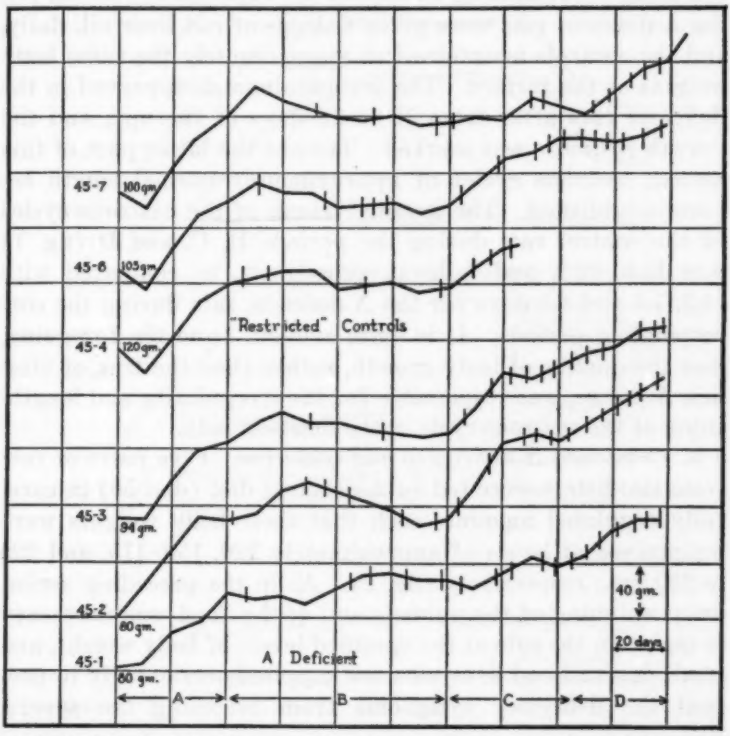

Fig. 1 Showing the frequency of oestrous cycles in vitamin A-deficient rats and in littermate sisters fed an adequate diet restricted in the amounts given such that their body weights approximated that of the deficient animals. A, period prior to taking of vaginal smears. B, period of chronic A-deficiency in rats 45-1 to 45-3. C, period of mild A-therapy in deficient rats. Sufficient to stimulate growth, but scarcely adequate to repair eye changes. Vertical lines represent the time of appearance of the oestrous phase of the cycle.

moderate growth response, but inadequate to completely repair the severe xeropthalmia. During the 43 days following (period C), the cycles were somewhat shortened; more so than in the 'restricted' controls, apparently due to the fact that the additional food supplied the latter was not sufficient to produce the same growth response as that exhibited by the deficient rats.

During the remaining 35 days of the experiment (period D), the A-deficient rats were given 6 drops of cod liver oil, daily, and the controls maintained at approximately the same body weights as the former. The xeropthalmia disappeared in the deficient rats after from 12 to 18 days of therapy, and the growth response was marked. Toward the latter part of this period, oestrous cycles of approximately normal length became established. The average length of the oestrous cycles of the control rats during the periods B, C, and D (fig. 1) was 15.3, 12.2, and 7 days, respectively, as compared with 14.3, 7.4 and 6.5 days for the A-deficient rats during the corresponding periods. It is quite evident, from the foregoing, that the quality of body growth, rather than the lack of vitamin A per se, was responsible for the irregularity and lengthening of the oestrous cycle in the deficient rats.

b. Combined A-deficiency and inanition. Five pairs of rats from two litters were fed an A-deficient diet (diet 56) in carefully regulated amounts such that their body weights were maintained at levels of approximately 120, 150, 175, and 225 to 250 gm., respectively (fig. 2). As in the preceding series, daily weighing of the animals and of the food was necessary to maintain the rats at the specified levels of body weight, and small doses of cod liver oil were supplied occasionally to prevent the deficiency symptoms from becoming too severe.

Fig. 2 Showing A, the effect of different levels of growth restriction on the frequency of oestrus in A-deficient rats (first 147 days). The lengthening of the oestrous cycles is in direct proportion to the severity of the growth retardation and independent of the other manifestations of A-deficiency. B, the effect of A-therapy associated with 1) gradual growth retardation induced by restriction of daily food intake (rats 40-73, -74, -76); 2) maintenance at same level of body weight (rats 40-67, -68, -71); 3) rapid growth on unlimited food supply (rat 40-69). Last 50 days.

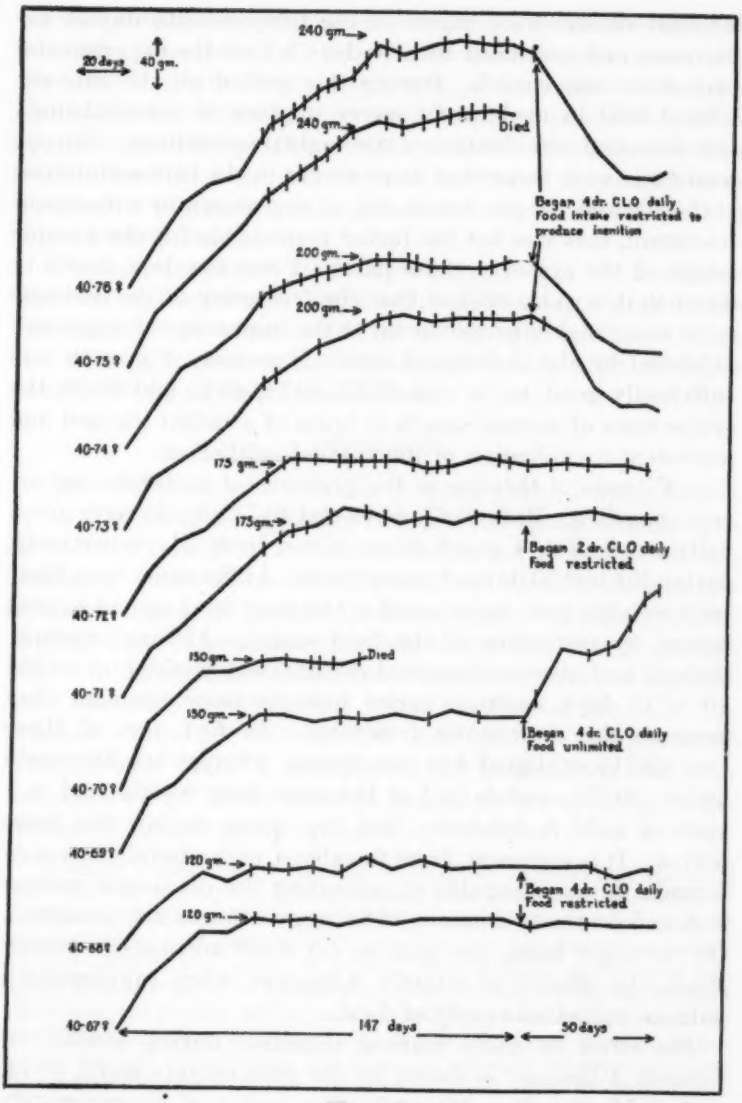
20 days
40 gm.
240 gm.
240 gm.
Died
Began 4 dr. CLO daily
Food intake restricted to
produce inanition
200 gm.
200 gm.
40-76 ♀
40-75 ♀
40-74 ♀
175 gm.
175 gm.
Began 2 dr. CLO daily
Food restricted
40-73 ♀
40-72 ♀
150 gm.
Died
40-71 ♀
150 gm.
Began 4 dr. CLO daily
Food unlimited
40-70 ♀
40-69 ♀
120 gm.
Began 4 dr. CLO daily
Food restricted
120 gm.
40-68 ♀
40-67 ♀
147 days
50 days

Figure 2

Vaginal smears were begun on the forty-seventh day of experiment and continued for 100 days before the experimental procedure was varied. During this period all the rats exhibited mild to moderately severe degrees of xerophthalmia and abnormal cornification of the vaginal epithelium. The eye conditions were somewhat more severe in the rats maintained at the 120 and 150 gm. levels, but, as was shown by subsequent treatment, this was not the factor responsible for the greater length of the cycles in these pairs. From the data shown in figure 2, it is quite evident that the frequency of the oestrous cycle was roughly proportional to the improvement in growth exhibited by the A-deficient rats. Moreover, if growth was sufficiently good, as in rats 40-73, 40-74, 40-75 and 40-76, the cycles were of normal length in spite of a rather marked and persistent cornification of the vaginal epithelium.

c. Vitamin A-therapy in the presence of moderate and severe inanition. Rats 40-67, 40-68 and 40-71 (fig. 2) were given daily doses of 4, 4 and 2 drops of cod liver oil, respectively, during the last 50 days of experiment. At the same time, their body weights were maintained at the same level as had existed before, by restriction of the food intake. Although xerophthalmia and abnormal vaginal cornification cleared up within 10 to 15 days, oestrous cycles were no more frequent than prior to the therapeutic treatment. In fact, one of these (rat 40-71) exhibited but two cycles, whereas its littermate sister (40-72), maintained at the same body weight and in a state of mild A-deficiency, had five cycles during this same period. It is apparent, from the above, that administration of vitamin A was incapable of correcting the prolonged oestrus in A-deficient rats if increased body growth was not permitted. On the other hand, the data on rat 40-69 adequately demonstrate the efficacy of vitamin A-therapy when supplemented with an unlimited supply of food.

The effect of more marked inanition during periods of vitamin A-therapy is shown by the data on rats 40-73, 40-74, and 40-76 (fig. 2). After showing cycles of approximately normal length for a period of 100 days, in spite of a mild

chronic xerophthalmia, these rats were given 4 drops of cod liver oil, daily, but their body weights were gradually diminished by a continuous reduction of the daily food intake. It will be seen that the degree of inanition was sufficient to completely suppress the oestrous cycles in these animals. After about 10 days, the vaginal smears of these rats consisted, for the most part, of small amounts of mucus and many leucocytes.

The data presented above demonstrate that the irregular and prolonged oestrous periods of A-deficient rats must be attributable to an indirect effect of the vitamin deficiency as reflected in the general nutritive state and quality of body growth of the experimental animals.

DISCUSSION

The studies presented in the present paper emphasize the importance of differentiating between the pathologic changes induced by lack of a particular vitamin per se and those due indirectly to other incidental conditions associated with the deficiency disease. In the alterations in the oestrous cycle of A-deficient rats, two separate factors have been clearly demonstrated. The specific effect due to lack of the vitamin factor is an excessive keratinization of the vaginal epithelium. The indirect effect, due to retarded body growth and generally lowered body state, is manifest in the irregularity and prolongation of the cyclic changes produced in the vaginal epithelium by hormonal influences.[3]

[3] A similar prolongation of dioestrus and increased lengthening of the entire cycle noted by Light ('27) in rats suffering from a deficiency of protein, of phosphorus and of vitamin A, seems best attributable to the general inanition of the experimental rats. Irregularity of oestrus has also been observed as a result of many other factors capable of inducing either hormonal imbalance or subnormal physical states of the experimental animal. Failure to recognize the effect of anorexia and physical debility upon the sensitive phenomenon of oestrus undoubtedly explains many of the contradictory statements in the literature regarding the effect of adrenalectomy, sympathectomy and other operative procedures upon the oestrous cycle.

Failure to recognize the importance of these two factors seems to best explain the failure of Parkes and Drummond ('26) to observe continuous cornification in the vaginal smears of their A-deficient rats, although they observed greatly prolonged cycles. Their animals usually weighed only about 40 gm. at 4 weeks of age, and rarely attained body weights of more than 100 gm. after 4 months of dietary treatment, and no mention was made of the existence of xerophthalmia. It would appear that their results were predominantly due to greatly retarded growth rather than to a direct effect of A-deficiency. It is apparently for the same reason that Coward ('29), and Coward, Morgan and Dyer ('30) were led to consider the vaginal cornification to be a rather unreliable criterion for diagnostic purposes.

We would like to stress the importance of using, for feeding experiments, animals which show unusually good growth and vigor during the weaning period. It has been our custom in most of our experimental work, to use only animals which have attained a body weight of 40 gm. or more at the twenty-first day of weaning. Utilization of inferior young has invariably led to atypical results, especially in studies concerned with the male or female reproductive system.

SUMMARY

1. The lavage method of obtaining smears of the cells of the rat's vagina, stained supravitally with neutral red, makes possible the demonstration of oestrous cycles in vitamin A-deficient animals in which abnormal cornification has become too severe to permit their identification by dry stained smears.

2. The excessive keratinization, which more or less masks the underlying cyclic changes, is a primary response of the vaginal epithelium to lack of vitamin A.

3. The increased lengthening of the cyclic change is indirectly due to associated growth retardation and decline.

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THE EFFECT OF RETARDED GROWTH UPON THE SEXUAL DEVELOPMENT OF RATS

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The study of longevity on a retarded diet recorded by McCay ('35) has afforded means of obtaining data on the sexual development of rats. The rats used in the study were albinos derived from the Mendel colony. Although no outside blood has been brought into our colony for several years, no more effort has been made to render the animals homozygous than is usual in a colony used for nutrition work so that it is probable that considerable genetic variation may be expected in the stock. However, the fact that litter mates were distributed among the three groups set up should make the degree of variation in each group similar.

A brief recapitulation of the methods and objects of the experiment so far as they affect us may be made. Three groups of rats were set up; the first or control group were allowed to grow as fast as they could upon an adequate diet, both quantitively and qualitatively. The second group were kept at a weight of 40 gm. and the third at a weight of 80 gm. until signs of failure occurred when both groups were allowed to rise 10 gm. in weight. This was continued until the rats were 766 days old when half the survivors were allowed an unrestricted diet, while at 911 days of age all the rats were unrestricted. The dietary deficiency may be regarded as solely one of calories. The animals were all kept isolated during the experiments; no matings were allowed so that the only observations which could be made were the time of opening the vaginal orifice and daily vaginal smears. After the

establishment of the vaginal canal, smears were taken daily until a rat showed six regular cornified smears at reasonably normal intervals, anything below 10 days being regarded as reasonable. Smears were then taken at 6-month intervals for a sufficient period, usually 6 weeks or more, to enable one to gauge the reproduction state of the animal at this time.

The circumstances under which the experiment was conducted allowed the following observations to be recorded.

1. Age and weight at the opening of the vaginal membrane.
2. Age and weight at first oestrus.
3. Regularity or otherwise of the oestrous cycles at intervals during life.

The results obtained under these headings are discussed separately for each group and compared.

The control group

These rats were allowed all they could eat of a qualitatively adequate diet. They may be assumed to have grown at an optimum rate and to have reached sexual maturity at the earliest age possible. We have no data on this point since it involves the ability to reproduce, which was not tested in this experiment.

Twenty-two rats were included in this group, but at the time the work was begun seven had open vaginas and their oestrous cycles were established. Also, one rat died immediately after opening, so her record was omitted from the record. The average age of opening for the remainder was 53.5 ± 0.19 days, the spread being between 49 and 59 days. The average weight was 136.7 ± 2.1 gm. with a variation from 120 to 165 gm. There is some evidence that the age of the rat is a more important determiner of the time of opening than the weight, but the number of cases discussed here is rather small. However, evidence pointing to the same result was obtained in the retarded groups so that this view is strengthened. If the rats in the normal group with ages of opening below the mean and those with ages above the mean are taken separately, the average weights of opening are 134.6 and 139.5 gm. respec-

tively, while for weights below and above the mean, the corresponding average ages were 53.6 and 53.4 days, a much closer agreement for the ages than for the weights.

We have been unable to find any records of the relation between age and weight at puberty for any species; all other records deal with age and the effect of various factors on the variability of the age. For rats, Evans and Bishop ('22 a) record that for 570 animals the average age at opening was 46.8 days, giving a considerably lower age for their strain, even allowing for the fact that some of our earlier opening rats were not recorded.

Our rats experienced their first oestrus, as judged by the vaginal smear, 1.8 days after opening, i.e., at 55.3 days. The delay in the Evans and Bishop rats was 0.5 day. One delay of 7 days greatly increased the average delay for our rats. On the whole, the behavior of the two strains is alike in this respect.

At the onset of their breeding powers all the control rats experienced regular cycles. In October 1931, when they were 11 months old, 21 rats of the group were alive; of these 13 were regular in their cycles, and 8 showed a continuously cornified smear. The average weight of the cornified rats was 275.9 gm. against 272.6 gm. for the normals.

In April 1932; age, 1 year 5 months, 19 survivors, 5 were regular, 10 cornified, 4 anoestrous. The normal rats weighed an average of 346.4 gm.; the cornified, 322.1 gm.; and the anoestrus, 375.8 gm.

In October 1932; age, 1 year 11 months, 12 survivors, 4 were regular, 1 cornified, 7 anoestrous. Average weights 297.3 gm., 320.0 gm., and 309.4 gm. respectively.

In April 1933; age, 2 years 5 months, 7 survivors, 2 were regular, 5 anoestrous. Average weights 263.0 gm. and 337.0 gm. respectively.

In October 1933; age, 2 years 11 months, there were 2 survivors, 1 being regular and the other anoestrous. Weights 280.0 gm. and 244.0 gm. respectively.

There is a marked tendency for the anoestrous rats to be heavier than those with regular cycles. The cornified rats are at first a little heavier than the regular ones, but, with increasing age, they lose this advantage. Probably the anoestrous rats experience the fattening which usually accompanies ovariectomy and are to be regarded as in a state of physiological ovariectomy. If the cornified rats are in a continuous state of oestrous activity, their relatively smaller weight is to be expected, but we have no data on the point, no activity studies having been made on our rats.

The frequency with which continued cornified smears occurred is surprising, 15 of 21 or 71.4 per cent showed a continued cornified smear at some time in their lives. This is probably due to the prevention of mating so that frequent cycles with ovulations occur. A thickening of the epithelium of the ovary and prevention of the rupture of follicles is a probable consequence. But we have no information on the number of rats which are allowed to reproduce which also show this continued cornification. This tendency is not inborn as a rule for all our rats had regular cycles at the beginning of their sexual life and some passed into this state later. Almost half the rats pass from the cornified state to an anoestrous condition while the other half tend to resume regular cycles. All patterns of behavior at the 6-month intervals were exhibited, i.e., cycles to cornified to anoestrum, cycles to cornified to cycles, and so on, except that when once a rat entered the anoestrous phase, she rarely showed further signs of sexual activity. So far as our experience shows, this condition is usually irreversible under our rather uniform conditions. Only one rat changed from cycles to anoestrum and then to cycles again. Her weight changes were 310 gm. to 460 gm. to 380 gm. which may be significant. While in the anoestrous state her weight was the greatest recorded during the experiment. Her return to the normal state was accompanied by a large loss in weight.

There is no evidence in our rats of a menopause at a given period of life such as is seen in man. The percentage of anoestrous rats at various ages was:

Age	*Number of rats alive*	*Per cent anoestrous*
11 months	21	0
1 year 5 months	19	21.1
1 year 11 months	12	58.3
2 years 5 months	7	71.4
2 years 11 months	2	50.0

Cessation of breeding activity under our conditions is a gradual process.

The 40-gm. group

The rats in the 40-gm. group were maintained at this weight until some of the retarded rats showed signs of failing or one died when the whole of the retarded groups were allowed to rise 10 gm. in weight. The diets used were adequate in protein, minerals and vitamins for growth at all ages but were deficient in calories. The method of raising the weight makes our data difficult to interpret since the slightest feeding of an extra amount of calories, or even a change in temperature leading to economy in utilization usually caused the animals to show a cornified or epithelial plus cornified smear (absence of leucocytes was used as the criterion of a heat period). Indeed this method was more accurate than the weighing of the animals as the histologist could often detect an oestrous smear before the balance showed a rise in weight. But, the rise having taken place, when the rats were maintained at a new weight level, cycles often ceased, so that often many weight increases took place, with a few cycles at each, before six regular cycles were experienced.

The weights at the time of the establishment of the vaginal orifice in these rats were much lower than in the rats of the other groups, but they were more uniform.

3 rats opened at 40 gm.

7 rats opened during the rise to 50 gm. or at 50 gm.

3 rats opened at 60 gm.

8 rats opened during rise to 70 gm. or at 70 gm.

On the other hand there was a much greater spread in the age of opening. The evidence shows that as the rats get older they open at a much lower weight than is normal. Age, therefore, is a more important consideration than weight in vaginal opening. The weight at opening in the three groups also declines progressively as the stunting is more severe, so that in the stunted groups, although the age at opening increases, opening does occur in spite of the lower weight.

The age at first oestrus averaged 357 days, and the weight 84.5 gm., a delay of 228.8 days and of 28.6 gm. over the controls. There is nothing in the evidence to indicate that capacity for experiencing oestrus is lost by the stunting. All the rats which died before their first oestrus were well below the maximum observed age at first oestrus, namely, 776 days.

The subsequent behavior of the rats is difficult to interpret. In all cases but one a considerable delay, usually from 200 to 300 days, was experienced before six regular cycles were recorded, and this usually happened when an extended rise in weight was occurring, so that there was time for the establishment of regular cycles.

At 1 year 11 months, 4 rats were regular the others decidedly irregular, not open, or not yet at the first oestrous period.

At 2 years 5 months, 8 were regular, 2 anoestrous after having been regular.

At 2 years 11 months, 4 were regular, 1 anoestrous, and 1 passing into anoestrum.

This is a better record than among the 80-gm. group, which, at this age, were all anoestrous or passing into this condition. None of this group showed a continued cornified smear at any time. This is in accordance with our experience in the 80-gm. group. We regard it as due to the smaller number of ovulations which had occurred in these retarded groups.

The 80-gm. group

The rats in the 80-gm. group were allowed to grow until they weighed 80 gm. after which they were kept at this weight until some failure occurred, when, as previosly mentioned, all the retarded rats were raised 10 gm. in weight.

When observations were begun one rat of this group had an opened vagina. She was not considered in working out the averages for age at opening, which makes these averages slightly too high. This rat was 61 days old when she was observed. The average for the opening of her class (those opening at 80 gm.) was 63.4 days.

Of the twenty rats in the group: 10 opened at 80 gm., 6 opened during the rise to 90 gm., 4 opened at 100 gm.

The first heat did not usually follow the opening of the vagina. In fact in most cases a delay occurred; sometimes a very considerable delay and a rise in weight did not produce a cornified smear though it usually did. The average age at first heat was 173.7 ± 21.1 days, and the average weight 104.4 ± 3.0 gm. This represents an average delay of 93.8 days and of 17.4 gm. in weight between opening and first oestrus.

After the first cornified smear the usual behavior was to show two or more cornified smears and then to remain quiescent until another rise in weight was made to occur. When six regular cycles were completed the rats were regarded as being in a healthy reproductive state. This state was reached in a very variable time; the average was 592.6 ± 30.2 days with weight 158.7 ± 4.39 gm. Six rats died before this stage was reached. After six regular cycles had been noted, the rats usually continued to show regular behavior, but tended to become irregular as they grew older.

At 1 year 5 months, 2 rats were regular, both weighing 150 gm.

At 1 year 11 months, 3 were regular, 1 irregular, 2 anoestrous, 3 had not reached six cycles.

At 2 years 5 months, 4 were regular, 1 irregular, 2 anoestrous, 2 had not reached six cycles.

At 2 years 11 months, 3 were irregular, 1 becoming anoestrous, 2 were anoestrous.

No rats in this series became continuously cornified, a condition which was also noted in the 40-gm. group.

A small autopsy group

In order to study the effects of this type of stunting on the reproductive tract, a group of six female rats was stunted by the methods used in the main experiment. These were killed at varying intervals.

A typical case is given here: Rat E63. Allowed to grow to 80 gm. and then allowed to rise gradually to 95 gm. Vagina opened at 78 days, weight 95.5 gm. steady. Killed at 106 days, no cornified smear. Reproductive tract infantile, uterine mucosa moderate in thickness, blood supply moderate and glands scanty. Ovaries, some follicular degeneration, moderate-sized follicles, no corpora lutea.

Three other rats with similar treatment produced a similar picture, but the degree of follicular degeneration was somewhat variable.

Rat E89. Killed 2 days after first cornified smear, 113 days after vaginal orifice established. Weight at killing 124.5 gm., rising. Uterus fairly well developed, glands moderate. Six recent corpora lutea were found in the two ovaries, but no ova were found in the tubes.

Rat E70. Killed 3 days after first cornified smear, 114 days after vaginal orifice established. Weight at killing 120 gm. on rise from 110 to 120 gm. Uterus fairly well developed, glands scanty. In the two ovaries were sixteen corpora lutea of at least two crops.

The results from these autopsied rats differ in no way from those which have been obtained by a number of other observers on the reproductive tract under the influence of inanition. The last case (E70) indicates that by our system of daily smearing occasional oestrous periods may be missed, though it is possible that with poor nutrition the full effects of ripening follicles on the vagina may not be exhibited.

The males

The conditions of the main experiment did not allow any experimental work to be done with the males. It was noted that all the males of the 40-gm. group developed a peculiar condition of the penis. This protruded from the sheath, and, instead of becoming pointed as in the normal adult, became bulbous with a flattened end. This prevented the return of the penis to the sheath and a condition resembling mild strangulation ensued, resulting in an aggravation of the condition. A similar condition occurred in a single male of the 80-gm. group and in none of the control group. The reason for the condition may be that male sex hormone was produced in these animals causing an hypertrophy of the penis at a time when the general body development was insufficient to accommodate it, thus resulting in a disharmony of the relative sizes of the parts (i.e., penis and sheath) and a distortion of the penis.

Two rats were grown under similar conditions to the experimental group and developed the condition of the penis described. They were killed when this occurred. Histological examination showed that the condition was due to oedema of the superficial layers of the corpus spongiosum which was well developed. The testes were almost normal, all layers of the seminiferous tubules were present and actual development of spermatozoa was occurring though not so frequently as in the controls. Only debris were found in the epididymes.

DISCUSSION

The only similar experiments on the effect of partial inanition on the development of sexual function in rats seem to be those of Evans and Bishop ('22 b). These workers explored the results of several types of partial inanition, including one which was entirely an energy deficiency. The results showed the same delay in the opening of the vaginal opening and the same dissociation between the opening and the first oestrous period. Subsequent cycles were highly irregular, with long

intervals between them. In one group which was maintained at from 60 to 80 gm. for 375 days, the vagina opened on the average at the ninty-ninth day of life and no oestrous cycle was observed during the period of observation. Our rats, even of the 40-gm. group did not show this extreme result for three experienced their first oestrous periods at 42, 43 and 54 gm. respectively, their ages being 377, 101 and 106 days respectively. Possibly our method of increasing the diet so that 10 gm. were gained at one time is the reason for the difference, but on the other hand we found the occurrence of oestrus an extremely sensitive indicator of a gain in weight. We were able to deduce as a general rule, that, when the food was increased, even slightly there was no competition for the increased energy between the growth and the sexual functions. Each received its share of the nutrients until the growth had reached a stage at which all the energy was required for maintenance when there was none to spare either for growth or for sexual needs.

At the present time it is a logical conclusion from the evidence available to deduce that both growth and the sexual function are controlled by the anterior hypophysis. In the normal young animal the hypophysis releases growth substance only, and when the genetic growth stimulus slackens, then sexual stimulating substance is also released in gradually increasing quantities. This accounts for the onset of puberty and the subsequent development of sexual maturity, which, as Crew ('30) has shown, is a gradual process. Our work described in this paper has shown that, so far as the opening of the vaginal membrane and incidence of the first heat period are concerned, after a time, the age influence becomes so great that it overcomes the genetic growth impetus and causes the release, when made possible by more food, of the sexual stimulating substance at a lower weight than is normal. In other words, there is an aging of the animal which determines the mode of functioning of the hypophysis.

SUMMARY

1. The oestrous behavior in the absence of mating was observed throughout life in a group of rats allowed to grow at at an unrestricted rate, in a group retarded for a considerable period at 40 gm. and then allowed to grow slowly, and in a group similarly treated after an initial weight of 80 gm. The limiting factor was that of energy in the diet.

2. The average age at the opening of the vagina is recorded, also the weight at this time, for each group.

3. The age of opening of the vagina increases with the increased severity of treatment and the weight at opening decreases. Two lines of evidence indicate that age is a more important consideration than weight in determining the time at which vaginal opening occurs.

4. Increased severity of treatment increases the age and weight interval between the vaginal opening and the occurrence of first oestrus. These two events become entirely dissociated in the retarded rats.

5. In the control rats the cessation of sexual activity is a gradual process, not abrupt as in man. It is usually irreversible.

6. Anoestrous rats tended to be heavier than rats with regular cycles.

7. Seventy-one per cent of the normal rats presented continued cornified vaginal smears at some time of their lives, while this never occurred in the two retarded groups. This is probably associated with the frequency of ovulation. Cornified rats tended to weigh less than those with regular cycles.

8. The occurrence of an oestrous smear is a very delicate indication of an increased supply of energy.

9. When more energy is supplied it is used for both growth and sexual needs; no competition is shown between these demands. But when growth has proceeded so far that all the energy available is needed for maintenance both growth and sexual activity cease.

10. The males of the 40-gm. group show a deformity of the penis, the etiology of which is discussed.

11. The bearing of 3 and 9 on the mode of action of the anterior hypophysis is discussed.

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STUDIES OF CRYSTALLINE VITAMIN B

IV. INJECTION METHOD OF ASSAY [1]

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ONE FIGURE

(Received for publication December 29, 1934)

It has been found advantageous in testing the potency of vitamin B(B_1) materials in this laboratory to use a variety of methods. In the earlier stages of work, when the emphasis was upon the resolution of vitamin B complex into its several factors, the activity of the fractions was determined by feeding experiments on rats and pigeons and later by feeding tests on rats only. However, during the past 2 years the principal objective has been the isolation of vitamin B(B_1) and for this purpose it was found advisable to adopt a quicker method of test as a guide for chemical procedures and one which is as specific as possible for the antineuritic factor alone. Smith's ('30) curative injection method presents an effective means of rapid assay. With slight modification it can be used on crude fractions. Over 2000 rats have been used in the testing of 500 preparations and the method has always proved reliable in estimating relative potencies.

The availability of crystalline vitamin B (Williams, Waterman and Keresztesy, '34) now affords an opportunity for noting the effect of specific doses of a single chemical entity and so calibrating this testing method.

[1] The first three papers in this series appear in J. Amer. Chem. Soc., 1934, vol. 56, p. 1187, 1935, vol. 57, pp. 517 and 536. In this series the designation B_1, preferred by the authors, has been used when consistent with the editorial policy of the journal involved.

Breeding and care of experimental animals

The rats used in this work were from a colony of albinos maintained on a stock diet consisting of one-third whole milk powder and two-thirds ground whole wheat with the addition of 1 per cent $CaCO_3$ and 1 per cent NaCl. About 1 gm. of raw beef was given to each rat three times a week. The young rats were kept on this diet until they weighed 100 to 125 gm. which occurred at 40 to 55 days of age. They were then placed in separate cages and given the following depleting diet:

	Per cent
Casein (technical)	16
Osborne and Mendel salt mixture	4
Cod liver oil	2
Unfiltered butter	9
Cornstarch	60
Autoclaved dried bakers' yeast	9

The dried bakers' yeast was autoclaved for 5 hours at 15 pounds' pressure at its natural pH, and then finely ground. This diet, which is a modification of the Sherman-Spohn diet, contains a small amount of the antineuritic vitamin. This has been found to be necessary to avoid inanition and to prolong the life of the rat sufficiently to permit the development of distinctive paralysis. Heyroth ('32) and Birch and Harris ('34) have also purposely used a depletion diet containing small amounts of vitamin B in their injection assays. This is contrary to Smith's view ('33), but he ignores the presence of small amounts of antineuritic vitamin which we (Williams, Waterman and Gurin, '32) have found to be present in autoclaved brewers' yeast such as he uses as part of his diet. The best polyneuritis-producing diet would be one supplying a precisely controlled small amount of B, from our experience an amount equivalent to about 0.5 γ per day of the crystalline vitamin. The rats gain in weight for about 2 weeks on the depletion diet, after which there is a gradual decline. In about 5 to 8 weeks, when the animals weigh 70 to 90 gm., paralysis occurs. This condition is characterized by incoordination, spasticity, and rolling movements of the head and

body. Of a group of 375 rats given this depletion diet, 70 per cent developed distinctive cases of paralysis. The remaining 30 per cent died without our observing paralytic symptoms and death was ascribed to inanition, urinary hemorrhage[2] or to paralysis so severe as to cause death before the animal could be used for test.

Technic

The material to be tested is injected from a tuberculin syringe through a 26 gauge needle into the fleshy part of the rat's hind leg. This procedure is more expeditious, requires less skillful manipulation than intravenous injection and only one operator is necessary. The solution is roughly adjusted to pH 4.0 to 6.0, but no especial care is taken in regard to salt concentration. As much as 0.75 cc. of liquid containing as high as 10 per cent solids can be introduced into each leg subcutaneously with satisfactory results. With high concentrations temporary soreness is noted at the point of injection. Lameness of the injected leg occasionally appears.[3] The effect of the injected material can be noted within 12 to 48 hours. When an adequate dose is administered, the symptoms of paralysis disappear entirely with an accompanying gain in weight. If the dose injected is grossly inadequate, the symptoms are not alleviated and may become definitely worse and there is generally a loss in weight. If the injected dose is barely adequate, a partial cure may result. In such border-line cases, the animals must often be reexamined and weighed on the third day in order to observe

[2] Occasionally, toward the end of the depletion period, some of the rats show severe hemorrhage in the urinary tract. Such a condition occurs in both males and females and is evidently not of genital origin. This hemorrhage, except in one case, has invariably proved fatal in a very few hours. Despite the fact that several animals may develop this condition at the same time followed by relatively long periods during which no cases are noted, we have no other reason to suspect that the disorder is infectious. It is not known whether this is a specific disease or one of the manifestations of extreme depletion.

[3] On the suggestion of Dr. Hans Molitor, of Merck & Co., subcutaneous injection in the back of the animal has been tried with marked success. The difficulty of distinguishing between leg soreness and paralysis is avoided and larger volumes can be injected.

retarded responses and so secure an indication of the degree of potency of the dose. With minimal curative doses paralysis recurs in 5 to 10 days after successful treatment. As a result of an extremely large dose, animals have remained cured for as long as 32 days. Surviving animals that are not cured by the first injection are used again after the lapse of 1 day. Those that are cured or definitely improved are kept on the depletion diet until paralysis recurs and are then reinjected. In this way the same rat can be used several times. Rats have been used for as many as ten successive tests with practically no change in response. A survey of injections during a given period of use of the method showed that 614 tests were made on 257 rats—an average of 2.4 tests per rat. Twenty-five per cent of the deaths followed trial doses which subsequent tests proved inadequate.

Individual response

A curative dose has arbitrarily been called the minimum amount capable of effecting cures of which at least two out of three shall be complete. A standard might with equal validity be set at which a dose would be the minimum amount completely curing every surviving animal injected. This would require more animals. According to our procedure, partial cures are helpful in obtaining reasonably precise estimations with a minimum number of injections.

A small percentage of the animals injected with doses, proved to be curative for other animals, dies within the first 24 hours. Such results are discarded as death is considered to be due not to the inadequacy of the dose but to a paralytic condition too advanced to be alleviated. It has been found that deaths of this sort can be eliminated to a great degree by the early detection and treatment of the paralytic symptoms. Sometimes a dose found curative for several animals produces mere improvement rather than cure in an individual rat; with such a dose completely negative results are never encountered in surviving rats. On the other hand, in no case has a rat recovered after receiving a preparation later found to contain little or no antineuritic vitamin.

The individual responses obtained from 181 injections of varying amounts of crystalline vitamin B hydrochloride are given in table 1. From these data it has been concluded that the curative dose according to our standards is 5 γ. Seven and one-half γ is the minimum dose which completely cures practically every animal.

Consistency of results

As in all forms of animal tests the precision of the method leaves something to be desired. The degree of consistency obtainable may be judged from the results on seven lots of pre-

TABLE 1

Individual responses on various dosages of crystalline vitamin B hydrochloride

DOSE (γ)	NUMBER OF ANIMALS			
	Cured	Partially cured	Not cured	Died [1]
2	0	2	2	3
3	2	4	0	2
4	8	10	3	12
5	33	17	2	20
6	10	8	0	2
7.5	17	2	0	6
10	9	0	0	7

[1] Discarded due to death in less than 24 hours.

sumably pure vitamin B (table 2). While relatively few tests have been made on some of the lots, 5 γ is considered the approximate dose in each case.

Duration of cure

Kinnersley, Peters and Reader ('28) have extensively used duration of cure as a means of evaluating the potency of antineuritic products. Their test animal is the pigeon and their 'day dose' is obtained by dividing the dose given by the number of days the bird remains free from polyneuritis. To determine whether such a method could be used in connection with the injection technic on rats, varying doses of a

single lot of crystalline B were administered to polyneuritic rats and the length of time before the recurrence of paralysis was noted. The results as given in table 3 show that the

TABLE 2

Consistency of results on various lots of crystalline vitamin B hydrochloride

LOT	DOSE (γ)	NUMBER OF ANIMALS			
		Cured	Partially cured	Not cured	Died [1]
A	4	1	0	2	0
	5	3	0	0	0
	7.5	1	0	0	0
	10	1	0	0	0
B	4	1	0	1	0
	5	2	1	0	1
	7.5	1	0	0	0
C	4	0	3	0	3
	5	5	2	1	5
	7.5	4	0	0	1
	10	8	0	0	7
D	2	0	2	2	3
	3	1	3	0	2
	4	4	1	0	0
	5	6	6	0	3
	6	6	2	0	1
	7.5	11	2	0	5
E	3	1	1	0	0
	4	0	2	0	2
	5	7	3	1	8
	6	1	0	0	1
F	4	2	4	0	7
	5	6	4	0	2
	6	3	2	0	0
G	5	4	1	0	1

[1] Discarded due to death in less than 24 hours.

duration of cure is a function of the dose but that the proportionality is far from exact. Our results are plotted in figure 1 and show a remarkable parallelism with the results

of Birch and Harris ('34) obtained from feeding various vitamin B preparations by mouth to depleted rats.

Usefulness of method. In the present state of uncertainty regarding the various factors in the B complex, feeding tests

TABLE 3

Effect of size of dosage of crystalline vitamin B on duration of cure

DOSE (γ)	DURATION OF CURE IN DAYS	
	Individuals	Average
5	5, 7, 6, 5, 8	6.2
7.5	5, 8, 10, 6	7.3
10	8, 9, 7, 13, 9, 8, 6, 12	9.0
15	9, 8, 9, 14, 11, 6, 13, 10, 14	10.4
25	13, 11, 15, 17, 14, 18, 14	15.3
35	19, 16, 17, 25, 18, 22, 27	20.6
50	23, 23, 26, 21, 30, 28, 32	26.3

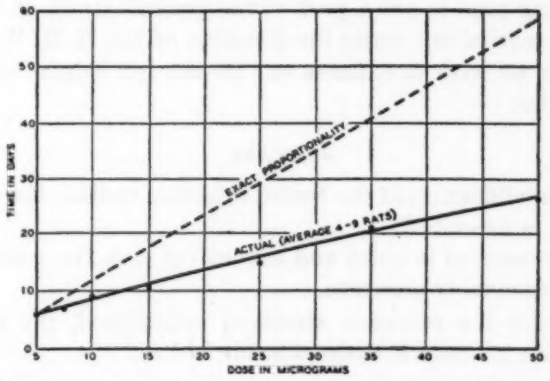

Fig. 1 Duration of cure of polyneuritic rats as a function of size of dose of crystalline vitamin B.

are necessarily open to question because the effect of over- or under-doses of any one of the other B factors may well have a profound effect on the rate of growth which is always used as a part of the critical data in such B assays. The injection technic on polyneuritic animals seemingly measures but one thing—the B content, and when the injections are

made subcutaneously relatively crude preparations can be used. This method is much to be preferred for the assay of therapeutic preparations. Liquid foods also are easily and accurately assayed in this manner. By developing a standardized extraction technic, it seems probable that practically all of the B in a solid foodstuff could be put into a solution suitable for injection. In this way a modification of the Smith injection method could be of practical use in the assay of foods, concerning the B content of which the present literature is grossly misleading on account of earlier failure to distinguish B from other growth promoting elements of the B complex.

The authors gratefully acknowledge generous financial assistance from the Carnegie Corporation of New York through the Carnegie Institution of Washington. This and the two succeeding papers are a part of the general study of vitamin B being carried out under the direction of Mr. R. R. Williams, to whom we wish to express our thanks for helpful criticism and advice.

SUMMARY

1. A modification of the Smith injection technic for vitamin B assay is described.

2. The method is quick and convenient and, for a biological assay, reasonably accurate.

3. Under the response standard established, the dose of crystalline vitamin B hydrochloride is 5 γ.

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STUDIES OF CRYSTALLINE VITAMIN B

V. THE EFFECT OF GRADUATED DOSES ON GROWING RATS

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SEVEN FIGURES

(Received for publication December 29, 1934)

Nutritional literature is replete with studies on the growth of young rats as effected by addenda of varying amounts of specific foodstuffs. There are few data on the growth attained when the addendum to a B-deficient diet is a vitamin in its pure form. The method of isolation of the antineuritic vitamin recently developed by Williams, Waterman and Keresztesy ('34) has made ample supplies of crystalline vitamin B hydrochloride available for study of the quantitative aspect of the effect of this vitamin in the diet.

EXPERIMENTAL METHOD

The animals used were from a stock colony of albino rats maintained on a diet consisting of two-thirds ground whole wheat and one-third whole milk powder plus 1 per cent $CaCO_3$ and 1 per cent NaCl with the addition of 1 gm. of raw beef per rat three times a week. At the age of 28 days, those rats which weighed 40 to 60 gm. were put in individual cages and given ad lib. the Chase modification of the Sherman-Spohn diet (Chase and Sherman, '31) consisting of 18 per cent B-free casein, 4 per cent Osborne and Mendel salt mixture, 2 per cent cod liver oil, 8 per cent butter fat, 53 per cent cornstarch, and 15 per cent dried autoclaved bakers' yeast. The bakers' yeast was neutralized with 0.1 N NaOH to pH 6.7 to

7.0, autoclaved at 15 pounds' pressure for 6 hours, dried at room temperature and finely ground. When possible, litter mates were put into test groups of two males and two females. After 15 to 17 days, the weights of the animals declined and addenda of crystalline B were started. The vitamin was fed every 5 days by mouth in amounts equivalent to daily doses ranging from 0.5 γ to 160 γ. (Studies are now in progress to determine the difference in growth attained by feeding addenda daily rather than in 5-day portions.) The crystals were dissolved in dilute alcohol and the solution pipetted into shallow dishes. The rats usually drink such a solution with avidity. Sometimes it was necessary to deprive the animals of water or to add a pinch of sugar to the solution in order to insure complete consumption of the supplement. The rats were weighed at 5-day intervals over a period of 45 days on the addenda. A control group of four rats was run on this diet whenever new lots of raw materials were used. Controls die in 28 to 40 days and rarely show typical paralysis, death being attributed to general inanition.

Growth on assay doses

Various investigators have proposed methods based on the rate of growth for assaying the B content of foods. Two standards now in use by which vitamin content is judged by growth rate are the Chase and Sherman ('31) and the Chick and Roscoe ('29). The former unit is that amount of vitamin B necessary to induce a gain of 3 gm. per week during an experimental period of from 4 to 8 weeks and the latter unit is that amount of vitamin B which results in a weekly rate of gain of 10 to 14 gm. The International unit is that amount of vitamin necessary to produce a rate of growth equal to that obtained by the daily administration of 10 mg. of 'International activated clay' to growing rats. Insufficient work has heretofore been done to indicate clearly the quantitative relationship of these different units of growth. It was therefore thought desirable to determine the relationship between the rate of growth and the size of dose under circumstances

where the basal diet is constant and the addendum consists of pure vitamin B without the complication of other factors. The results obtained in the dosage range of the usual B test will be discussed first.

All the animals in two groups of four fed at the level of 0.5 γ of crystalline B hydrochloride died in less than 45 days with true paralysis. The behavior of these animals as contrasted to that of the control rats on the B free diet which died without paralysis is regarded as evidence for the necessity of including small amounts of B in diets designed for the production of typical polyneuritis (Ammerman and Waterman, '35). One γ was the lowest dosage on which all of a

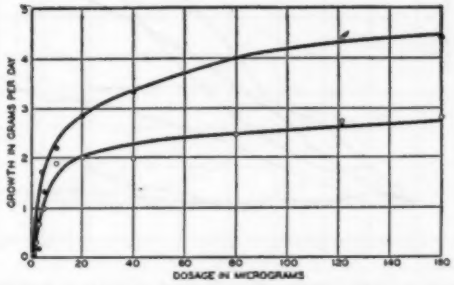

Fig. 1 Growth rate of young rats as a function of the size of dose. The period covered is 45 days following a 15- to 17-day depletion of 28-day-old rats. The dosage is the daily amount of crystalline vitamin B hydrochloride each rat received in 5-day portions during the 45-day test period.

single group of four rats survived the test period. Sherman-Chase unit growth occurred between 2 γ and 3 γ (fig. 1). Definite cases of paralysis have been encountered in animals receiving 1 γ and even 2 γ daily and one death, possibly due to B deficiency, occurred on the 3 γ dosage. The 4 γ level is the lowest which showed no suggestion of deficiency disease, the growth being about 1 gm. per day. Chick and Roscoe unit growth is not attained until a level somewhere between 5 and 10 γ is reached. On rather incomplete evidence, we have provisionally set the B content of the Chick and Roscoe unit as 7.5 γ. In comparison, 10 mg. of International standard

clay daily gave, with our diet, an average growth of 0.8 gm. per day for two males and two females, corresponding to the 5 γ level of crystalline vitamin. In the preceding paper of this series, the curative dose by the Smith injection technic was set at 5 γ. Each of the better known units may now be stated in terms of one another and also in terms of pure

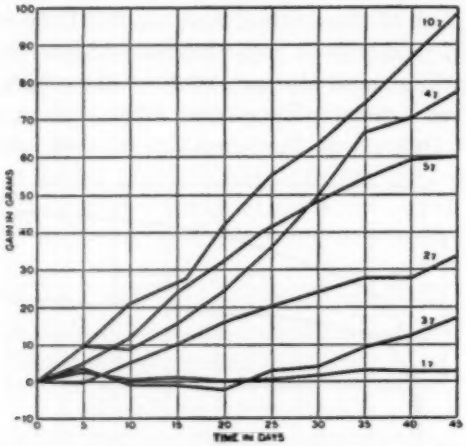

Fig. 2 Average growth curves of male rats receiving moderate amounts of crystalline vitamin B. The addenda were given every 5 days. The chart shows the intakes of vitamin calculated on a daily basis. The addenda were started after 28-day-old rats had been depleted 15 to 17 days on a B free diet.

vitamin B hydrochloride. This tentative equivalence is as follows:

1 Chick and Roscoe unit	= 7.5 γ	= 3 Sherman-Chase units
1 Smith unit [1]	= 5.0 γ	= 2 Sherman-Chase units
1 International unit	= 5.0 γ	= 2 Sherman-Chase units
1 Sherman-Chase unit	= 2.5 γ	

[1] Under an interpretation of the Smith method which sets the curative dose as that amount of vitamin which effects a cure in every successful injection, the unit would be approximately 7.5 γ or three Sherman-Chase units.

However, attention is called to a certain lack of consistency in the growth response of different groups of our animals in spite of the fact that our stock colony has been inbred and maintained on the same diet for many years. This incon-

sistency is more conspicuous over the lower range of dosages (figs. 2 and 3) and reflects itself in variation from animal to animal at any given level, as indicated in table 1. This inconsistency of growth response is a weakness in any method of assay which depends on mere measurement of growth and is partly circumvented by the use of the International method providing the growth on an unknown is compared at the same

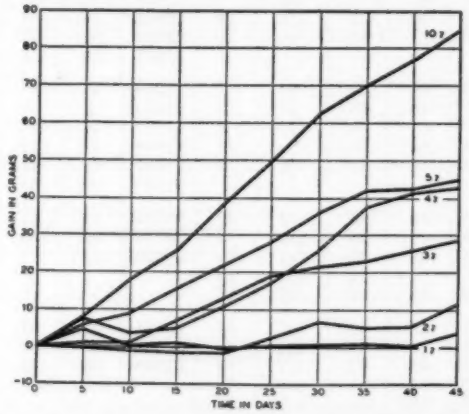

Fig. 3 Average growth curves of female rats receiving moderate amounts of crystalline vitamin B. The addenda were given every 5 days. The chart shows the intakes of vitamin calculated on a daily basis. The addenda were started after 28-day-old rats had been depleted 15 to 17 days on a B free diet.

time with growth on the standard with matched litters under identical conditions. This presupposes, of course, that the standard is constant. It will be more satisfactory to set crystalline B as the standard when enough of this material becomes available for universal calibration.

Duration of cure

At the end of the test period the surviving animals were given the polyneuritis-producing diet used in our injection assays (Ammerman and Waterman, '35) and the length of time before the onset of paralysis or death was noted. These data appear in table 1 and the average duration of time before

TABLE 1

Actual weights of individual animals

DOSE (γ)	MALES					FEMALES				
	No. of rat	W_1	W_2	W_3	E	No. of rat	W_1	W_2	W_3	E
0.5	23431	40	62	D	D	23282	45	61	D	D
	23280	44	69	D	D	23279	48	76	D	D
	23281	44	76	D	D	23417	48	67	D	D
	23399	42	68	D	D					
	23397	45	62	D	D					
1.0	22771	45	53	45	3	22774	42	54	47	4
	22772	44	57	56	6	23807	40	63	69	11
	22773	44	55	48	4	23808	46	63	76	21
	23805	45	60	78	8					
2.0	21620	55	71	79	6	21621	47	40	45	4
	22094	46	74	95	9	21622	54	50	D	D
	22095	43	68	128	9	21623	56	64	57	2
	22096	44	73	118	13	22085	48	56	70	12
	22610	50	59	93	11	22602	52	65	76	13
						22607	59	81	121	15
3.0	22070	43	51	76	7	21298	48	49	76	21
	22071	45	45	65	3	21319	45	49	101	N
	21747	54	69	77	8	21320	46	51	96	23
	21748	56	70	D	D	22084	48	54	72	11
	21749	51	62	72	5	22303	44	46	46	8
						21750	52	68	66	10
4.0	22498	54	87	136	20	22504	51	68	144	24
	22499	46	68	180	22	20319	46	45	74	10
	22501	58	75	161	24	20320	57	58	83	10
	20317	53	51	123	19					
	20318	52	54	123	17					
5.0	20862	46	64	105	34	20864	43	61	94	38
	20863	42	61	128	49	21567	49	66	122	34
	20865	46	63	117	39	21568	51	72	119	17
	21565	54	81	140	18					
	21566	55	86	163	N					
10.0	21313	55	61	154	26	21314	49	56	116	24
	22604	59	79	170	26	21315	54	51	138	30
	22603	59	72	172	30	21316	49	55	117	28
	23125	44	65	137	24	22605	49	62	147	42
	23184	53	82	198	24	22608	62	84	182	32
						23126	42	64	147	36
						23186	43	51	169	47
20.0	21318	48	63	207	31	21336	49	53	131	32
	21333	53	54	177	27	21335	48	55	140	24
	23232	51	76	216	44	23234	49	83	164	44
	23233	47	69	180	28	23235	47	74	186	57
40.0	21728	47	63	222	38	21726	48	52	140	38
	22080	49	60	193	30	21727	48	50	140	30
	22081	55	68	219	40	21729	49	47	132	29
	22082	55	65	219	40	22083	48	56	139	49
80.0	22321	40	66	253	58	22324	41	66	176	48
	22322	40	62	249	43					
	22323	40	57	218	40					
160.0	22500	52	71	252	C	22503	52	69	190	C
	22502	55	77	292	C	22505	50	56	185	C

D = Animal died before completion of test. C = Animal being continued beyond normal test period. N = Data not available.

Column W_1 = Weight at weaning (28 days old). Column W_2 = Weight at start of test (28 to 33 days old). Column W_3 = Weight at end of test (73 to 78 days old). Column E = Time in days between end of test and onset of paralysis or death.

death or paralysis of the several animals at each dosage level is given graphically in figure 4. In this curve the duration of time is the number of days between the last dose of vitamin and the onset of polyneuritis or death and the dosage given is the actual amount of vitamin administered at 5-day intervals. The results indicate that the duration of time bears a straight line relationship to the size of dose up to about 25 γ. Beyond that level, the curve bends indicating a poor conservation of surplus vitamin in the animal organism.

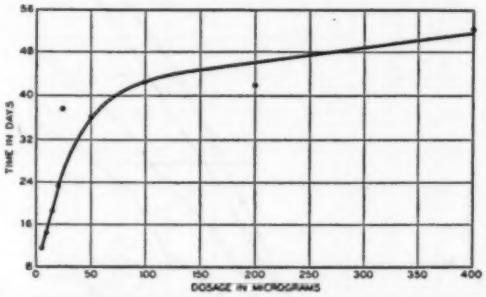

Fig. 4 Duration of effect in relation to size of vitamin B dosage. The time is the period from the last administration of the vitamin to the onset of paralysis or death. The dose is the actual amount of crystalline vitamin received.

Growth on relatively high doses

The literature contains many indications that a superior performance ensues from the administration of amounts of vitamin B larger than those requisite for the prevention of polyneuritis. There are also numerous suggestions, especially in the exploitation of commercial vitamin-containing products, that still larger amounts of vitamin contribute to general well-being. However, concrete evidence has heretofore been lacking that the effect of multiplication of the dosage extends far beyond the customary range of intakes. The continued upward sweep of the growth curves in figures 5 and 6 as the dosage levels range through 10, 20, 40, 80 and 160 γ constitutes evidence of a remarkable stimulation of metabolic processes as reflected in growth. The rats receiving 160 γ per day were continued on addendum beyond the

regular 45-day experimental period. Figure 7 gives the growth results on these animals up to an age of 233 days.

In these experiments there is little possibility that enhanced performance can be due to other vitamin factors in the ad-

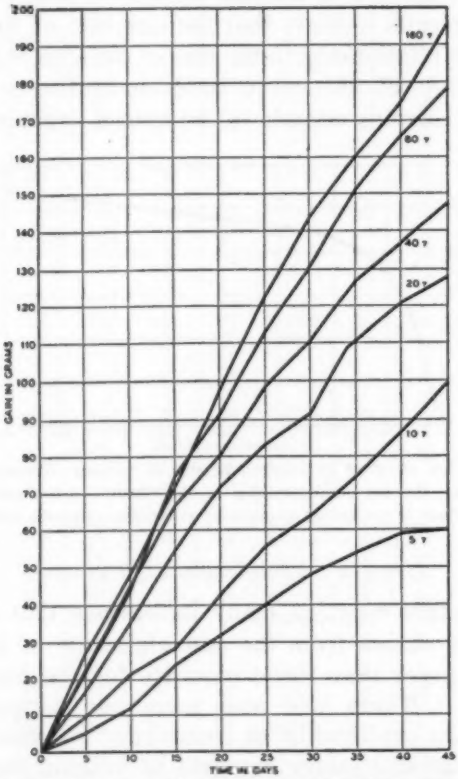

Fig. 5 Average growth curves of male rats receiving large amounts of crystalline vitamin B. The addenda were given every 5 days. The chart shows the intakes of vitamin calculated on a daily basis. The addenda were started after 28-day-old rats had been depleted 15 to 17 days on a B free diet.

dendum as is the case when the addendum consists of a crude product. Vitamins A, D, G, etc., which quite possibly are present in sub-optimal amounts remain constant increasing only with the food intake yet the growth at the higher

levels of B dosage approaches the best obtainable with rich mixed diets (Osborne and Mendel, '33). A possible explanation of this remarkable growth stimulation is that the crystals contain B_4 as an impurity. This is not believed to be the

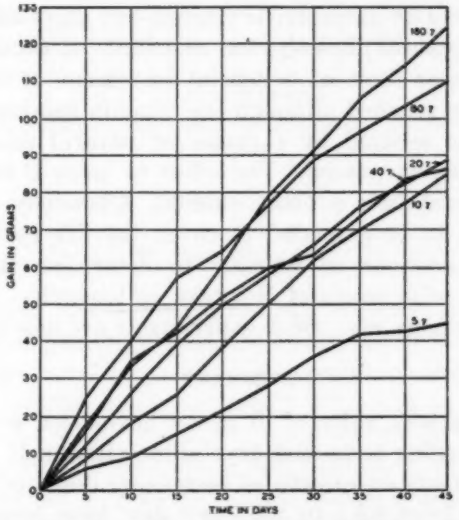

Fig. 6 Average growth curves of female rats receiving large amounts of crystalline vitamin B. The addenda were given every 5 days. The chart shows the intakes of vitamin calculated on a daily basis. The addenda were started after 28-day-old rats had been depleted 15 to 17 days on a B free diet.

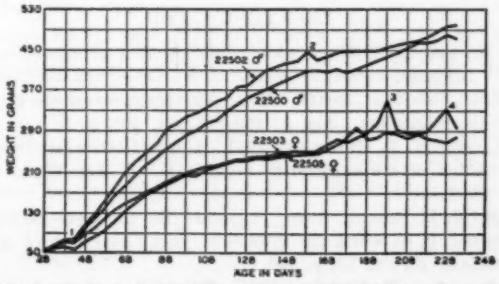

Fig. 7 Weight curves of individual rats receiving 160 γ of crystalline B per day. At point 1 the administration of the vitamin in 5-day portions was started. At point 2 no. 22502 was mated with 22503 and no. 22500 with 22505. At points 3 and 4, nos. 22503 and 22505 had litters of ten and nine young, respectively.

case. A more probable explanation is that large amounts of B exercise a growth acceleration sometimes confused with that due to B_4.

These results raise several profound questions with regard to the physiological action of this vitamin. It is obvious that B is not solely an antineuritic vitamin but plays an important role in the general physiological economy. It should be noted that the upper limit of beneficial increment of the vitamin has not been reached although the vitamin intakes which can generally be secured by a choice of natural foods has apparently been surpassed. The effect of large B supplements to rich natural diets is being studied. Obviously, the experiments need to be extended to cover the life cycle and successive generations of animals to determine the ultimate effects of a wide range of B intake on longevity, size, reproductive functions, etc. Such experiments are now in progress.

SUMMARY

1. The various units of B assay have been compared by means of feeding tests with crystalline vitamin B.

2. The effects on growth of increasing doses of crystalline B, ranging from 0.5 γ to 160 γ per day, have been observed.

3. The stimulative effect persists up to and probably beyond a dosage of 160 γ, an amount 80 to 160 times that necessary for maintenance of life.

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FACTORS AFFECTING THE CAROTENE CONTENT OF ALFALFA HAY AND MEAL[1]

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ONE FIGURE

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Recognition of the importance of vitamin A in commercial livestock and poultry production has resulted in considerable work on the variation in vitamin A value of alfalfa hay and meal, the sources most commonly employed to supply this food essential. The literature, most of which has been reviewed recently by Scheunert and Schieblich ('34), is, however, somewhat confusing. The valuable contribution of Russell ('29), which showed that a sample of artificially dried alfalfa contained at least seven times as much vitamin A as a field-cured sample that had lost most of its green color, became widely quoted, and unjustifiable, broad generalizations were made on the basis of this figure. Dehydration plants were built and the products sold on the assumption that all samples of artificially dried alfalfa contained seven to ten times as much vitamin A as field-cured products. Although most subsequent tests have shown greater vitamin A potency in machine dried than in field-cured hays, the relative values have been variable and in at least two cases (Kiesselbach and Anderson, '31; Guilbert, '34) no significant difference has been found.

[1] This report is part of an investigation on the relation of nutrition to reproduction in livestock which became cooperative with the U. S. Bureau of Animal Industry, July 1, 1929.

In 1931, Hauge and Aitkenhead reported on the effect of artificial drying upon the vitamin A content of alfalfa. They stated in their discussion, "These experiments clearly demonstrate that the practice of treating alfalfa at elevated temperatures in mechanical dryers is without deleterious effect on vitamin A. Even a temperature as high as the hot flue gas used in one of the machines, or the sterilization temperature of the autoclave, was not destructive." They further stated, "that the sun's rays (ultra-violet rays) are not responsible for the destruction of vitamin A during the field curing process." These authors concluded also that enzymes are the important factor in the destruction of vitamin A during curing. Later experiments (Hauge, '34) gave essentially the same results. The implication that artificially dried alfalfa is the equivalent of fresh green forage does not appear to agree with observed results under commercial conditions nor does it agree with the work of Sherwood and Fraps ('32) and of Fraps and Treichler ('33). Scheunert and Schieblich ('34) also assumed that there was little or no loss of vitamin A in artificial drying, but no adequate means was employed to test the original material.

In a recent paper (Guilbert, '34) some of the most important work, justifying the use of carotene analyses as a means of determining vitamin A value of forage has been summarized. The accumulated evidence leads to the conclusion that the vitamin A problem in alfalfa is essentially a carotene problem. Carotene is well known to be readily oxidized and at least since the time of Capranica (1877) has been recognized as a light-sensitive compound. These facts alone would appear to cast some doubt upon the conclusions of Hauge and Aitkenhead ('31) which minimized the importance of sunlight and oxidation. Our own data showed extremely rapid destruction of carotene in dry alfalfa meal when exposed to sunlight (under glass) which could not be accounted for by enzyme action nor by ultra-violet rays. It was consequently decided to investigate further the factors affecting the carotene content of alfalfa hays and meals. The technic

used for the carotene determinations is described in a previous publication (Guilbert, '34).

EXPERIMENTAL

Smith and Milner ('34) have shown that practically all of the carotene in alfalfa leaves is the optically inactive beta form. The carotene values presented in this report are expressed, therefore, on the basis of beta carotene. The dye solution used in the colorimetric determinations was standardized against a sample of beta carotene kindly furnished to us by Dr. H. H. Strain, Division of Plant Biology, Carnegie Institution of Washington, Stanford University, California. We have previously reported carotene values of alfalfa upon the basis of International Standard carotene. The values reported here may be converted to the International Standard basis by multiplying by the factor 1.12.

Unless otherwise stated the samples of alfalfa were cut and brought immediately to the laboratory. Less than an hour elapsed between the time of cutting and the time the sample was either in process of analysis or undergoing the treatment specified in subsequent tables. Dried samples that could not be analyzed at once, were placed in stoppered tubes and stored at a temperature below 0°C., a condition shown to be adequate to prevent deterioration. All analyses are calculated to the moisture-free basis.

Effect of vacuum-drying. Four tests were run comparing the carotene analyses of fresh alfalfa leaves, and samples of the same leaves after vacuum-drying for 3 hours at 100°C. The carotene values obtained from the fresh leaf samples were 48.9, 52.0, 46.6 and 60.3 mg. per cent, respectively, while the values on the corresponding vacuum-dried samples were 48.4, 51.3, 46.6 and 57.1 mg. per cent. Thus there was little or no loss in vacuum-drying three of the four samples and the apparent loss in the fourth sample was 5.3 per cent. These results are in agreement with the well-known fact that carotene (and vitamin A as well) is relatively stable to heat in the absence of oxygen.

Since vacuum-drying is more convenient and assures more accurate sampling of the small amount required for carotene determinations than fresh leaves, all reference or control samples have been vacuum-dried for 3 hours at 100°C.

Effect of autoclaving for 1 hour at 17 pounds pressure. Hauge and Aitkenhead ('31) indicated that autoclaving did not diminish the vitamin A potency of alfalfa. The value of their original material, however, was not determined. Presumably no loss would occur in an autoclave from which the air could be rapidly evacuated. In our experiments a horizontal Bramhall Deane gas-heated autoclave was used, the inside dimensions of which were 26 × 36 inches. Two to 4 pounds of freshly cut alfalfa were used in each test. The time required for the temperature to approach the theoretical value for 17 pounds pressure was 15 to 20 minutes. In a series of four tests the vacuum-dried control samples contained 57.1, 57.4, 58.1 and 47.9 mg. per cent of carotene, respectively. The corresponding samples, autoclaved, then vacuum-dried contained 18.6, 25.0, 38.7 and 25.3 mg. per cent of carotene, the loss varying from 33 to 67.5 per cent. Tests on two of the samples showed that all of the chromogenic substances in the unsaponifiable fraction that react with antimony trichloride to form a blue color, decreased in proportion to the carotene.

Effect of sun-exposure. In table 1 are presented the results of several tests on the effect of sun-drying, and of sun-exposure on dried samples. Reference to test 1 in the table shows a loss of 69.5 per cent of the carotene in a sun-dried leaf sample taken from whole plants that were exposed in a layer approximating the thickness of hay in the swath. The leaves were practically dry in a few hours so that the period during which enzyme action might occur was relatively short. A vacuum-dried sample covered with ordinary glass and exposed to the sun at the same time decreased in carotene content almost as much as the sun-dried sample. The vacuum-dried sample, under glass, was subjected during most of the period of sun-exposure to a temperature averaging

about 65°C. or 15°C. higher than that of the sun-dried sample. To check the effect of the temperature difference, a portion of the control sample was subjected to a temperature 65° in the dark for 12 hours. This sample decreased in carotene content only 6.8 per cent. Since there was little difference in the rate of destruction in the sun-dried sample and the

TABLE 1

Effect of sun-exposure on the carotene content of alfalfa leaves

TEST NO.	TREATMENT	CAROTENE, MILLIGRAMS PER 100 GM. (DRY BASIS)	PERCENTAGE LOSS
1	Leaves, vacuum-dried (control)	57.1	
	Leaves, sun-dried. Twelve hours sun-exposure, taken into laboratory 28 hours after cutting	17.4	69.5
	Leaves, vacuum-dried then exposed to sunlight the same as above, except that it was under glass	18.6	67.4
	Leaves, vacuum-dried, then exposed for 12 hours in the dark to a temperature of 65° in the presence of air	53.2	6.8
2	Leaves, autoclaved then vacuum-dried (control)	18.6	
	Leaves, autoclaved then sun-dried the same as in test 1	8.6	53.8
3	Leaves, vacuum-dried (control)	58.1	
	Leaves, sun-dried 12 hours taken to laboratory 27 hours after cutting	21.1	63.7
4	Leaves, autoclaved then vacuum-dried (control)	38.7	
	Leaves, autoclaved then sun-dried as in test 3	26.3	32.0
5	Leaves, vacuum-dried (control)	47.9	
	Leaves, sun-dried, 6¼ hours semi-cloudy, 7½ hours bright sunlight, taken to laboratory 28 hours after cutting	25.7	46.4
6	Leaves, autoclaved then vacuum-dried (control)	25.3	
	Leaves, autoclaved then sun-dried as in test 5	18.6	26.5
7	Dehydrated alfalfa leaf meal (control)	12.3	
	Sample after 8 hours sun-exposure under glass	6.0	51.2
	Sample after 16 hours sun-exposure under glass	3.6	70.7
	Sample after 40 hours sun-exposure under glass	2.1	83.3

previously dried sample when exposed to the sun during the same period of time, it appears that, under conditions favorable for rapid drying, temperature and enzyme action were minor factors compared to the photo-chemical activation of the destructive process. Furthermore, the sample shown in test 2 which was autoclaved to stop enzyme action, suffered a loss of 53.8 per cent of its carotene when exposed to the sun simultaneously with the samples of test 1.

Tests 3, 4, 5, 6 and 7 in table 1 offer additional data on the loss from sun-drying and from sun-exposure of previously dried samples. These data show that autoclaving to prevent enzyme action reduced, but by no means stopped the destruction of carotene during sun-drying, and that contrary to the conclusions of Hauge and Aitkenhead ('31) the sun's rays play an important part in the destruction of vitamin A potency during field curing. That ultra-violet rays are not necessarily involved, is shown by the fact that destruction of carotene proceeds rapidly in samples covered with ordinary glass. In tests 3, 4, 5 and 6 the rate of sun-drying was slower than in tests 1 and 2 and the apparent loss from enzyme action (the difference in percentage loss between fresh and autoclaved samples) was greater.

Effect of enzyme action. Further study of the effect of enzyme action on the carotene content of alfalfa leaves is presented in table 2. In the dark, at room temperature (20° to 30°C.), some process in the living plant tissue caused a loss of 8.5 per cent of the carotene in 24 hours and 54.1 per cent in 75 hours. A sample from hay exposed over-night in a windrow and to sunlight in the late evening and early morning for about 4 hours, showed a loss of 10.2 per cent of its carotene.

A sample of leaves was placed over water to which was added a small quantity of toluene and kept in an incubator room at a temperature of 38°C. The air in the incubator was practically saturated with moisture. These conditions are somewhat similar to those in the experiments of Hauge and Aitkenhead ('31). At the end of 24 hours the alfalfa

leaves had the dark olive-drab color of autoclaved samples and 95 per cent of the carotene had been lost. There was no evidence of mould, but it appears doubtful that this technic would be effective in eliminating bacterial action. An autoclaved sample under the same conditions lost 33 per cent

TABLE 2

Effect of enzyme action on the carotene content of alfalfa leaves

TEST NO.	TREATMENT	CAROTENE, MILLIGRAMS PER 100 GM. (DRY BASIS)	PERCENTAGE LOSS
9	Leaves, vacuum-dried (control)	57.1	
	Leaves, after standing in dark at 20 to 30°C. for 24 hours without wilting, then vacuum-dried	52.3	8.5
	Leaves, after standing in dark at 20 to 30°C. for 75 hours without appreciable wilting, then vacuum-dried	26.2	54.1
	Leaves, after standing from 5 P.M. to 8 A.M. in the field in a windrow then vacuum-dried	51.3	10.2
	Leaves, placed over water and toluene for 24 hours at 38°C., then vacuum-dried	2.9	94.9
10	Leaves, autoclaved then vacuum-dried (control)	18.6	
	Leaves, autoclaved then placed over water and toluene for 24 hours at 38°C., then vacuum-dried	12.4	33.3
11	Leaves, vacuum-dried (control)	47.9	
	Leaves, exposed at 38°C. over water and toluene for 24 hours, then vacuum-dried	24.5	48.9
12	Leaves, autoclaved then vacuum-dried (control)	25.3	
	Leaves, autoclaved then incubated and vacuum-dried in test 11	25.3	0.0

of its carotene (test 10). It appears, therefore, that enzyme and possibly bacterial action was important in the rapid destruction of carotene in the incubated fresh leaf sample of test 9.

The results of a second trial are shown by tests 11 and 12 in table 2. In this experiment a sample of fresh leaves and a sample of autoclaved leaves were placed in open desiccators

over water and toluene and were kept in an incubator at 38°C. for 24 hours. The air in this incubator was relatively dry, causing slight wilting of the fresh leaf sample during the night. The autoclaved sample remained wet throughout the 24-hour period. The fresh leaf sample, which remained green in color, lost 49 per cent of its carotene under these conditions while there was no loss in the autoclaved sample.

TABLE 3

Effect of temperature on the loss of carotene in alfalfa during storage

TEST NO.	TREATMENT	CAROTENE, MILLIGRAMS PER 100 GM. (DRY BASIS)	PERCENTAGE LOSS
13	Leaves, sun-dried (control)	17.4	
	Sample after 8 weeks storage at 0 to 5°C.	17.9	...
14	Leaves, vacuum-dried (control)	57.1	
	Sample after 8 weeks storage at 0 to 5°C.	56.9	...
15	Sun-cured alfalfa meal (control)	6.6	
	Sample after 8 weeks storage at —5 to 0°C.	6.7	...
16	Dehydrated alfalfa meal (control)	13.6	
	Sample after 8 weeks storage at —5 to 0°C.	13.2	...
17	Sun-cured alfalfa meal (control)	7.9	
	Sample after 8 weeks storage at 20 to 30°C.	5.5	30.4
18	Dehydrated alfalfa meal (control)	16.7	
	Sample after 8 weeks storage at 20 to 30°C.	11.6	30.5
19	Fresh leaves, vacuum-dried (control)	55.4	
	Sample after 9 days in the dark at 60°C.	21.1	61.9
	Sample after 9 days in the dark at 80°C.	7.3	86.8

These data indicate that during warm cloudy weather, or when moist hay heats in the cock or stack, loss of carotene from enzyme action may be rapid. Bacterial action under these conditions should also be considered.

Effect of environmental temperature on the loss of carotene during storage. Fraps ('33) and Sherwood and Fraps ('32) have demonstrated by biological tests, that the vitamin A potency of natural feeds deteriorates during storage.

Russell, Taylor and Chichester ('34) found that there was no loss of carotene in alfalfa samples stored in vacuo at 0° ± 5°. These authors also indicate that the rate of loss of carotene in dried plant tissue may be greater during the first few months of storage than after a longer period, while data from Fraps and Treichler ('33) indicate the opposite.

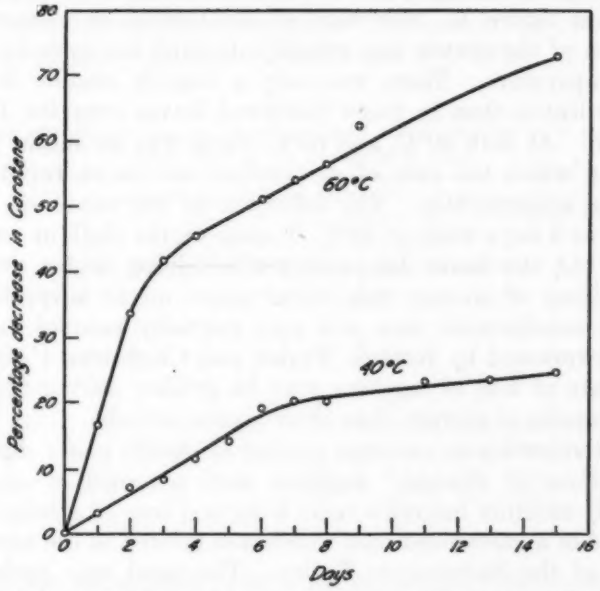

Fig. 1 Percentage decrease in carotene content of vacuum-dried alfalfa leaves stored in the dark at temperatures of 40° and 60°C., respectively.

Data on the destruction of carotene in alfalfa at different temperatures are presented in table 3. There was no loss in samples either in stoppered tubes, or in heavy paper sample containers, when stored for 8 weeks in the dark at temperatures of —5° to 5°C. (tests 13, 14, 15 and 16). There was a 30 per cent loss of carotene in alfalfa meals stored in the dark, in paper sample containers, for a period of 8 weeks at room temperature (about 20° to 30°C.) (tests 17 and 18).

Test 19 shows the effect of exposure in the dark for 9 days at temperatures of 60° and 80°C., respectively. Oxidation undoubtedly being the cause of the carotene loss, acceleration of the rate at higher temperatures, is to be expected.

The percentage decrease in carotene content of finely ground alfalfa leaves placed in ovens at 40°C. and 60°C., respectively, during a period of 15 days is presented graphically in figure 1. The rate of destruction at comparable phases of the curves was roughly doubled for each 10° rise in temperature. There was only a slightly smaller loss in whole leaves than in finely powdered leaves over the 15-day period. At both 40°C. and 60°C. there was an initial phase during which the rate of destruction was more rapid than it was subsequently. The inflection of the curve at 60°C. came at 3 days while at 40°C. it came on the sixth or seventh day. At the lower temperatures obtaining under average conditions of storage this initial phase might be prolonged for a considerable time and may partially account for the idea expressed by Russell, Taylor and Chichester ('34) that the rate of loss of carotene may be greater during the first few months of storage than after longer periods.

Deterioration in carotene content of alfalfa under different conditions of storage. Samples were obtained at approximately monthly intervals from 5-ton test lots of alfalfa meal, stored in a galvanized iron warehouse located in the northern part of the Sacramento Valley. The meal was packed in burlap bags containing 100 pounds each. The results of the carotene analyses together with the mean monthly temperatures are shown in table 4. The temperature record was obtained from a U. S. Weather Bureau station located 10 miles distant, but at about the same elevation and the temperatures of the two places are similar. No record on the temperature inside the warehouse was available, but it was stated that the temperature was not uncommonly 10°F. lower than outside air temperature. The temperature of the alfalfa meal after dehydration, grinding and packing into 100 pound bags was usually about 100°F. Hence, it is reasonable to

assume that the mean temperature of the meal during the period of storage investigated was 70°F. or higher.

While some inconsistencies appear in the results, which are probably caused by error in sampling, serious losses unquestionably occur under these conditions. Dehydrated meal after several months of storage may be much lower in carotene than freshly prepared sun-dried meal, carefully cured under favorable conditions. The rate of loss declined during the cool weather in October and November.

TABLE 4

Decrease of carotene content of alfalfa meal during storage in a warehouse from June 26 to December 6, 1934

DATE	CAROTENE, MILLIGRAMS PER 100 GM.					MONTHLY TEMPERATURE U. S. WEATHER BUREAU	
	Lot 1 Dehydrated	Lot 2 Dehydrated	Lot 2 Sun-cured	Lot 3 Dehydrated	Lot 3 Sun-cured	Mean	Highest
						°F.	°F.
June 26	11.3	11.4	7.9			June 76	113
July 12	...	...	...	16.7	7.9		
July 27	8.9	6.9	7.1	...	...	July 81.2	109
Aug. 8	...	...	...	13.2	6.6		
Aug. 27	6.3	5.9	7.0	...	...	Aug. 82.3	108
Oct. 1	...	4.6	4.1	...	...	Sept. 77.3	107
Oct. 9	7.1	...	...	10.3	5.1	Oct. 65.0	100
Nov. 7	5.8	4.5	3.9	6.5	4.8	Nov. 54.2	77
Dec. 6	5.7	3.9	4.9	6.7	4.8		

Samples were taken from a lot of chopped, sun-cured hay that was stored in a dark loft at the University Farm, Davis. The values obtained in November, 1933, April, 1934 and August, 1934 were 3.3, 3.0 and 2.1 mg. per cent of carotene, respectively. During the comparatively cool period from November to April the decrease was about 9 per cent, while during the hot weather from April to August the decrease was 30 per cent.

While other factors than temperature may be involved in changing rates of deterioration, as previously indicated, it appears probable that temperature variations may explain the discordant results previously reported. Thus if meal goes

into storage in the spring, comparatively rapid loss may be expected during the summer months and little loss during the succeeding fall and winter. If, however, the alfalfa is stored from late summer or fall crops, little loss might be expected during the following cold months and heavy losses during hot weather of the next spring and summer.

Seasonal variation in carotene content of dehydrated and sun-cured alfalfa meals. A series of samples was obtained from a ranch in the Sacramento Valley that produces both sun-cured and dehydrated alfalfa meals. The alfalfa all came from the same ranch and the sun-cured and dehydrated lots from which the test samples were taken were in each case cut at the same time and from adjoining checks in the same field. Because the capacity of the dehydrator was greatly increased by a 10 to 15 per cent reduction of moisture in the field, and because of the practical difficulties of supplying hay to the dehydrator immediately after cutting, the hay had more or less exposure in the field before dehydration. In some cases the hay was cut, raked within an hour and hauled to the dehydrator a few hours after cutting. In other instances the hay was cut in the late afternoon, raked into windrows and left in the field over-night, to have a supply ready for the dehydrator in the morning. The dehydrator employs, in the process, flue gases along with considerable excess air. The chopped hay is first blown into the hot gas stream at the base of a verticle, funnel-shaped tower where it is floated out of the top as moisture is lost. The hay remains in this part of the dehydrator a maximum of 30 seconds where the temperature of the flue gases at the base ranges from 900° to 1200°F. depending on the moisture content of the hay. The drop in temperature is extremely rapid. Next it goes through a revolving, horizontal drum at a temperature varying from 230° to 270°F. The whole process requires about 15 to 18 minutes. The leaves and finer particles, however, go through the dehydrator in 2 to 3 minutes.

In this area the relatively low humidity and high temperatures throughout the summer are favorable for rapid sun-curing. The hay was carefully handled to facilitate drying

and to minimize sun-bleaching. The period elapsing between the time of cutting and milling varied from 2 to 5 days. Usually it was ground and sacked on the fourth day. A typical example of the sun-curing process is as follows: "The hay was cut, raked into windrows and cocked on July 19. The cocks were turned July 21, loaded on trucks the evening of July 22 and milled the morning of July 23."

The hay, throughout the season, was cut after 3 to 4 weeks of growth and the samples were received and analyzed within a week after curing. The data are presented in table 5.

TABLE 5

Seasonal variation in dehydrated and sun-cured alfalfa meals

DATE OF CUTTING	DEHYDRATED ALFALFA MEAL, CAROTENE MILLIGRAMS PER 100 GM.	SUN-CURED ALFALFA MEAL, CAROTENE MILLIGRAMS PER 100 GM.
May 24, 1934	11.3	...
June 21, 1934	11.4	7.8
July 5, 1934	16.7	7.9
July 19, 1934	13.7	8.2
August 9, 1934	15.7	8.5
August 23, 1934	18.4	7.3
September 13, 1934	20.9	12.1
October 3, 1934	22.2	12.1
October 29, 1934	29.6	...

In both the sun-cured meals and the dehydrated, but in the latter particularly, there was a marked trend toward higher carotene values from spring to autumn. The variables that may have a bearing on these results are so numerous that definite interpretation is impossible. There is good evidence, however, that considerable loss occurred between the time of cutting and dehydration. This loss would depend on temperature, enzyme action, and intensity of sun-exposure during the variable time elapsing between cutting and dehydrating. Decrease in loss before dehydrating caused by cooler weather and less intense sunlight may have contributed significantly to the higher values obtained in the fall. Improvements in field handling that tended to minimize exposure

during periods of most intense sunlight were also initiated during the latter part of the season. Furthermore, differences caused by variation in the stage of maturity of the plants must be considered. The last cutting, for example, because of slower growth, was more immature when harvested than earlier cuttings. The following data are pertinent to the question of the influence of stage of maturity upon the carotene content of the plants.

Analyses of vacuum-dried stems and leaves at various stages of growth have given carotene values for the stems varying from 6 to 11 mg. per cent while the leaves varied from 45 to 60 mg. per cent. Data collected by the Division of Agronomy, University of California, on three varieties of alfalfa (Madson, '31) shows that the growth 15 days after harvesting consisted of about 60 per cent leaves and 40 per cent stems. At 22 days the proportion was about equal and at 30 days' growth the proportion was 55 per cent stems and 45 per cent leaves. It appears, therefore, that proportion of stems to leaves, hence stage of maturity, would play a dominant role in determining the carotene content of the plant. Hauge ('34) found the highest vitamin A value in young alfalfa 10 to 12 inches high and he further found by biological tests that leaves were ten to fourteen times as potent as the stems. The highest values for vacuum-dried whole plants, which we have thus far obtained, have been from immature plants 10 to 12 inches high cut early in November. The values both from the fields from which the October 29th sample in table 5 came, and from similar growth on the University Farm were from 39.4 to 42.7 mg. per cent. The data on stage of growth and possible seasonal variation in relation to carotene content are yet too meager to warrant conclusions. That the carotene content of the original plant material is one of the factors influencing the variation in the carotene content of dehydrated meals shown in table 5, is, however, strongly indicated.

Evidence of loss during mechanical dehydration. Most of the fresh samples of dehydrated alfalfa meals that we have analyzed contained from 13 to 22 mg. per cent of carotene.

The highest values reported by Russell, Taylor and Chichester ('34) were from 18 to 22 mg. per cent. The values obtained for vacuum-dried whole plants cut at various stages of growth have varied from 27 to 42 mg. per cent. This circumstantial evidence would lead one to the conclusion that serious losses must occur during mechanical dehydration. In some instances losses of carotene between the time of cutting and dehydration and also storage losses must be considered.

Russell, Taylor and Chichester ('34) found no loss in dehydration, in fact they obtained slightly lower carotene values on the fresh samples of alfalfa than for the dehydrated. They state, however, that they had some difficulty in getting the fresh material into a finely divided state for extraction and that some loss may have occurred between the time of harvesting and analysis. This time was not stated except that the samples were analyzed 'within a week.'

Direct evidence regarding loss in dehydration was obtained from one series of samples secured from the ranch previously mentioned, the data from which are shown in the following tabulation:

	Carotene milligrams per 100 gm.
Sample at time of cutting, 3 P.M., October 25	41.3
Sample when delivered at dehydrator, 10.30 A.M., October 26, after standing in windrow over-night	36.6
Sample of dehydrated meal	33.9

The samples taken at the time of cutting and on delivery at the dehydrator were placed in sealed mason jars and kept at O°C. until delivered at the laboratory 24 hours after cutting. They were then immediately vacuum-dried and stored in the cold until analyzed. The percentage loss in the field in this test was 11.4 per cent and the loss in the dehydrator was 7.4 per cent. The total loss in these operations was 18 per cent of the original carotene.

The results of one test should not be considered conclusive because of possible errors in sampling. The data indicate, however, that losses occur in mechanical dehydration. Incidentally the value of the meal in this test (33.9 mg. per cent) is the highest we have encountered.

DISCUSSION

Factors influencing the carotene content of alfalfa hays and meals have been studied by means of carotene determinations, using a simplified technic for routine analysis described in a previous publication (Guilbert, '34).

Little or no loss occurred in vacuum-drying, a finding in agreement with the well-known fact that carotene is relatively stable to heat in the absence of air.

Autoclaving resulted in significant losses of carotene presumably because the air could not be rapidly exhausted from the chamber of the autoclave used in these experiments. Losses apparently occurred also in the experiments of Hauge ('34), as the highest value reported for leaves that were autoclaved and then sun-dried was 140 Sherman units per gram. On the basis of present standards this value would be equivalent to approximately 12 mg. of carotene per 100 gm.—a comparatively low value for alfalfa leaves.

Extremely rapid loss of carotene results from sun-drying or from exposing dried alfalfa to either direct sunlight or sunlight filtered through ordinary glass. Autoclaving to stop enzyme action reduced but by no means eliminated loss of carotene during subsequent sun-drying.

Enzyme action was found to be an important factor in loss of carotene during the curing process. Under favorable conditions for sun-drying, however, enzyme action and temperature appeared to be minor factors compared with photo-chemical activation of the destructive process. Under unfavorable conditions for drying, prolonged enzyme action coupled with other destructive processes may result in hay with very low carotene content.

Temperature was shown to be the major factor causing variation in loss of carotene in alfalfa hay or meal during storage. No loss occurred over a period of 8 weeks at —5° to 5°C. while losses of 30 per cent occurred during a similar time at 20° to 30°C. The loss during 9 days at 60°C. and 80°C. was 62 and 87 per cent, respectively. Thus the rate of loss increases as the temperature rises, tests over 15 days

at 40° and 60°C., respectively, indicating that the rate of loss is roughly doubled for each 10° rise in temperature. Large losses of carotene occurred in dehydrated and sun-cured meals stored in a warehouse during hot weather. After 3 or 4 months dehydrated meals may be significantly lower in carotene than freshly prepared sun-dried meal, carefully cured under favorable conditions.

The carotene content of dehydrated alfalfa meals analyzed in this laboratory and also that reported by others are markedly lower in carotene than samples of whole plants cut at various stages of growth and vacuum-dried. This difference may be accounted for by losses in the hay before delivery to the dehydrator, losses during dehydration and by losses during storage.

CONCLUSIONS

The accumulated evidence summarized in a previous publication (Guilbert, '34) leads to the conclusion that the vitamin A problem in alfalfa is essentially a carotene problem.

The variability in carotene content of alfalfa hays and meals, both dehydrated and sun-cured, is so great, not only because of destruction during curing and storage, but also because of variation in the original hay as affected by stage of maturity, etc., that no sweeping generalities should be made regarding the relative values depending on method of curing.

Recognition of the various factors affecting the carotene content of alfalfa hay and meal should make possible a reasonable degree of standardization of products prepared especially for their vitamin A value. Such standardization would necessarily be subject to time limitations, however, unless effective means are employed to prevent deterioration in storage.

Feed dealers and cooperatives who are buying large quantities of alfalfa meals to incorporate into rations primarily as a source of vitamin A would do well to consider purchasing on the basis of carotene content, since the analytical technic is no more complicated, nor expensive than the other feed analyses commonly made.

ACKNOWLEDGMENT

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THE EFFECT OF RETARDED GROWTH UPON THE LENGTH OF LIFE SPAN AND UPON THE ULTIMATE BODY SIZE [1]

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ONE FIGURE

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In a preliminary report, the literature concerning the effect of retarded growth upon the life span was reviewed (McCay and Crowell, '34). In this report was also included a summary in the nature of a progress report dealing with a study employing rats to determine the effect of retarding growth upon the total length of life.

The present summary represents a complete, final report of this experiment employing white rats and covering a period of nearly 4 years. The object of this study was to determine the effect of retarding growth upon the total length of life and to measure the effects of retarded growth upon the ultimate size of the animal's body. In the present study, growth was retarded by limiting the calories.

The growth of an animal can be retarded either by disease or by various nutritional deficiencies. Every laboratory that performs vitamin assays is familiar with the retarded growth and prompt death that result when there is a deficiency of a certain factor such as vitamin A. In such experiments the animal grows little and dies prematurely. However, a borderline level of such an essential as vitamin A may permit a very slow growth and permit the animal to approach or attain

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adult size. Under such conditions the question arises as to the effect of this retarded growth upon the life span and ultimate body size. In the present study we have retarded the growth by restriction of calories, but with a diet designed to provide adequate levels of all other constituents.

In our previous report some of the earlier literature dealing with the interrelationship between the rate of growth and the length of life, was reviewed. The existence of such a relationship has been stressed again very recently by Kermack and associates ('34) in the following words:

> It is shown that these results are consistent with the hypothesis that the important factor from the point of view of the health of the individual during his whole life is his environment up to the age of say 15 years and that improved conditions at later ages have little direct effect.

This is a recognition that factors during the growing period are paramount in their influence upon the subsequent life span.

A few attempts have been made in other laboratories to test the hypothesis that a slow rate of growth results in an increased life span, by feeding a given species, such as mice, the same diet, recording the rate of growth and finally the length of life. At the conclusion of such an experiment the rates of growth and the length of life have been correlated. On the basis of such a study Robertson and Ray ('20) concluded that mice that grew the more rapidly lived the longer. They also concluded that the group that grew the slower was more unstable. Sherman and Campbell ('34) report, however, that rate of growth and length of life of rats on the same diet, vary independently of each other. Earlier, Campbell ('28) found that an improvement in the diet resulted both in increased rate of growth and increased length of life in the same individuals.

It is doubtful if such studies as those of Robertson really test the hypothesis, because the two groups, separated on the basis of growth, are not homogeneous. The slower growing group tends to include the inferior individuals that die prematurely. A genuine test of this hypothesis can be made,

however, if animals are separated into groups at the time of weaning or shortly thereafter. Such groups can be forced to grow at different rates and the length of life can be determined. In this latter case we are dealing with more homogeneous samples. Random selection provides reasonable insurance of similar expectancies of life spans and rates of growth in such groups, if other factors are constant.

EXPERIMENTAL

At the present time it seems that the only method of determining the interrelationship between the rate of attaining maturity and the total length of life is by direct experiments upon homogeneous groups of animals. For this reason 106 white rats were divided into three groups at the time of weaning. One group contained thirty-four individuals and the other two thirty-six. Group I was allowed all the feed desired and grew normally. Group II was restricted in feed intake from the time of weaning. Group III was allowed sufficient feed to permit normal growth for 2 weeks after weaning and then restricted in the same manner as group II.

These animals were confined in false-bottom cages such as those used for vitamin assays except that individuals tended to develop sore feet from time to time as old age approached. In these cases they were placed upon a solid bottom covered with shavings until the feet healed.

A diet was desired that would provide an excess of all recognized essentials for rapid growth except sufficient calories.

The diet designed for these experiments was a synthetic mixture of starch 22, cellulose 2, lard 10, sucrose 10, salt mixture 6, dried yeast 5, cod liver oil 5 and casein 40. The starch was cooked and dried. The cellulose was the regenerated product described elsewhere (McCay, '34). The salt mixture was that of Osborne and Mendel. The casein was not purified.

This diet was made high in all dietary essentials in order that the retarded-growth rats with restricted daily intakes

might have adequate protein, minerals and vitamins. In order to further compensate for the possible shortage of vitamins in the retarded growth groups, an additional 3 drops of cod liver oil and 0.5 gm. of yeast were fed daily to each member of groups II and III. This was estimated to compensate for the vitamins in the larger amounts of diet ingested by the rapidly growing group, no. I.

In the use of this diet which is rich in essentials such as protein, it is recognized that the group ingesting food ad libitum, may be subject to injury by the excess above the requirements of the body. It is not likely that such injury was produced in the present case, however, since the animals that matured rapidly had life spans similar to those found previously in our colony upon stock diets.

In a study of retarded growth the animal can be retarded for a long period and then allowed to grow. Osborne and Mendel ('15) employed this method. An optional procedure consists in holding the animal at a constant weight for a period of weeks, then allowing it to make a slight gain at a normal growth rate and following this in turn by another period of constant body weight. This procedure was used in an earlier study with brook trout (McCay, Dilley and Crowell, '29). This 'stairstep' method was employed in the present study with rats.

The feed for each animal of the retarded growth groups was weighed separately each day. The individuals were weighed three times weekly. The allowance of feed was thus adjusted to hold the body weight of each member of the retarded groups as nearly constant as possible. Usually a growth of 10 gm. was permitted to each individual of the retarded groups at intervals of 2 to 3 months. The period of holding them at a constant weight was determined by the appearance of the animals. As soon as any members of the retarded groups seemed to be failing from the deficiency of calories the entire group was allowed to grow to the extent of the 10 gm.

Two different methods were employed during the period of 10 gm. growth. Part of the time an allowance of sucrose was given in addition to the usual maintenance allowance of feed. Growth became normal in every case showing that the diet was adequate except for calories. In most cases, however, fresh beef liver was fed during this growth period. An equal allowance of this liver was given the animals of group I.

At the time the surviving rats were 766 days old, the retarded-growth groups were each subdivided. Half of each group was given all the feed it desired. The other half in each case was continued on the restricted intake until the 911th day. From that period all animals were allowed all the diet desired. The purpose of this subdivision was to determine if any difference in the power to resume growth existed between the 766th and the 911th days. Osborne and Mendel ('15), in the case of one rat, found growth was resumed after retardation for 552 days. After this retardation the rat attained a weight of 204 gm. and they stated that this was 'full size' for the female of this species.

In figure 1 are plotted the growth curves for the three groups. The curves at the top for group I show the rates for males and females separately. In the case of the retarded-growth animals the growth rates of the two sexes were maintained the same until the time for the resumption of growth. The ends of these curves show clearly that the male rat grows more rapidly and attains a larger size than the female even after growth has been retarded for more than 900 days. The power to grow still exists in the rat body after this period of 900 days.

At the top of figure 1 are shown the number of each sex alive in each group at various times. It will be observed that about forty rats were alive at the time of the first division on the 727th day.

These growth curves indicate that the retarded-growth rats attained about the same final weight as those of Osborne and Mendel, namely, 200 gm., but by comparison with the growth

curves for group I it is evident that the 'normal' size was not reached by the animals of the retarded-growth groups.

In table 1 are summarized the data regarding the weights attained which indicate the final size of the animal in terms of body weights.

In group I only animals that reached an age of 177 days or more are included. In groups II and III only those that

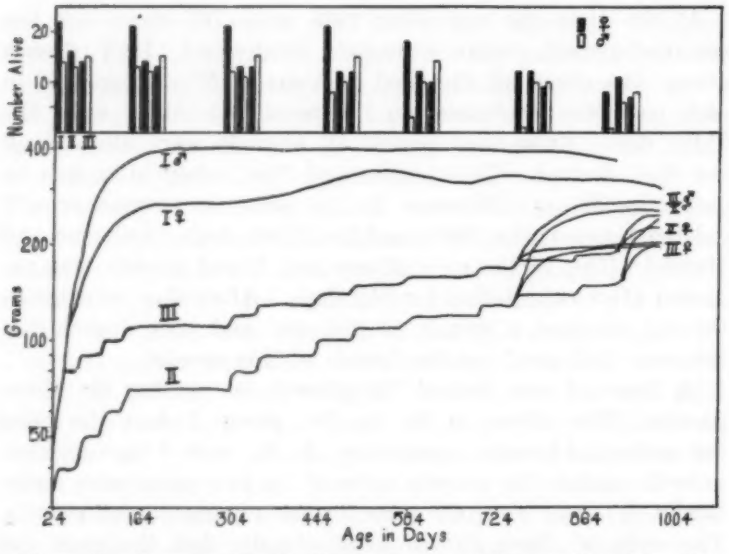

Fig. 1 Growth curves for rats allowed to grow rapidly, no. I, and for those retarded in growth by limiting the calories, nos. II and III. The columns at the top show the numbers alive by sex and group at various periods of the experiment. The top of these columns provides a mortality curve.

survived to make the final growth with ad libitum feeding are considered. These data are striking in the uniformity of the final weights of the retarded animals. The males of these retarded animals are significantly heavier than the females. Thus the undeveloped male of the species retains its potential growth power at a higher level than the female even after the male has lived for a period equal to nearly twice the average life span for this sex.

In terms of weight the difference between the final size of the animals that grew normally is significantly higher than those of the retarded groups. It is evident that animals retarded in growth for the long period that we have used are unable to attain the same final weight as those that grew rapidly to maturity. The tendency of white rats to attain a greater body weight than they did 20 years ago has been

TABLE 1

Maximum weights in grams attained by groups and sub-groups. 'G' signifies the half of the group allowed to finish growth after 766 days and 'R' after 911 days

GROUP	MEAN MAXIMUM WEIGHT ATTAINED	MEAN AGE OF ATTAINING MAXIMUM WEIGHT	MAXIMUM WEIGHT ATTAINED BY ANY INDIVIDUAL	NUMBER OF ANIMALS CONSIDERED
	gm.	*Days*	*gm.*	
I ♂	439±10	359±25	542	12
I ♀	355± 8	588±17	474	21
II ♂	262±13	1005±25	352	7
II ♀	210± 5	951±12	244	13
III ♂	267±12	903±17	358	9
III ♀	227± 5	918±26	260	10
IIG ♂	264±22	998±40	352	4
IIR ♂	259±17	1013±31	288	3
IIG ♀	223± 4	936±21	244	7
IIR ♀	195± 7	968± 8	233	6
IIIG ♂	266±20	851±18	358	5
IIIR ♂	268±13	968± 6	316	4
IIIG ♀	212± 5	840±18	238	6
IIIR ♀	256± 3	1036±27	260	4

observed in such laboratories as those at Yale. This suggests that there may be a close relation between the early growth rate and the final size since it is well recognized that the better knowledge of nutrition and selection have made it possible to stimulate growth and final size beyond the rates attainable 20 years ago. Our data suggest that a body of a certain size can be attained after very long periods of retarded growth, but that the attainment of the maximum size possible for a given species is impossible if growth is not permitted during early life. This is shown in table 1

by the attainment of the same maximum weights by groups II and III but both sexes of III g, the half of the group allowed to grow at 766 days, attained the maximum weights more quickly since they were larger when growth was permitted. They did not grow heavier, however, than those of group II.

The length of life for each individual of each group is given in table 2. The greatest length attained by any individual was 1421 days. There were members of each of the retarded-growth groups, nos. II and III, alive after all the members of group I had died. These data indicate clearly that some factor tended to promote longevity in the case of groups II and III. This effect is much more marked in the male sex than in the female. In two preceding reports (McCay and Crowell, '34; McCay, '34) it has been noted that the male rats of our colony tend to live an average of about 500 days.

The average life span of the male rats in the retarded-growth groups was 820 days in one case and 894 in the other. Upon the best diet among five, Slonaker's ('31) male rats averaged 767 days at the time of death while those of Campbell ('29) averaged 635 days upon her best diet. No male rat in Slonaker's recent experiments attained an age of 1200 days while one male among each six members of this sex of our retarded-growth groups exceeded this age. The males of our retarded groups tended to exceed the females in length of life.

The average life span of the females of the three groups is probably the same. No marked extension results from retarding the growth of this sex as far as our data indicate. Our data are somewhat distorted, however, due to the loss of two members of group II very early in the experiment. These were lost during a period of extremely hot weather. The best females in Slonaker's experiments lived an average of 848 days while the better of our retarded female groups averaged only 826 days. On the other hand, no female animal exceeded an age of 1250 days in Slonaker's study while four

of our retarded females, or about 10 per cent, exceeded this age. The average age of Miss Campbell's female rats on her better diet was 664 days. It is difficult to provide an explanation for this span of Campbell's animals which is so much shorter than either those of our own colony or those of Slonaker. Before studying the data of Slonaker it seemed

TABLE 2

Life span of individuals in days

GROUP					
I ♂	I ♀	II ♂	II ♀	III ♂	III ♀
71	74	41	48	548	134
162	485	249	62	580	341
338	520	313	259	602	479
354	559	758	260	650	565
397	699	758	260	723	577
401	713	780	296	832	641
499	719	797	342	845	663
544	722	964	410	919	810
561	724	1006	755	978	822
563	733	1137	887	998	894
588	817	1244	901	1022	901
627	824	1294	904	1028	920
733	855	1321	925	1168	999
927	857		938	1218	1080
	870		980	1306	1111
	984		999		1123
	997		1041		1132
	1023		1107		1210
	1039		1225		1297
	1074		1232		
	1143		1261		
	1189		1304		
			1421		
483	801	820	775	894	826

that reproduction might have shortened the span of Miss Campbell's females, but this is hardly tenable when we consider that Slonaker's females also reproduced.

Since half of each retarded group of rats was allowed to complete its growth after 766 days and the remaining half retarded until after 911 days, it was thought that some difference in life span might result. The number of animals

alive at the time final growth was permitted was too small. The great variability in life spans of this limited number of cases made it impossible to find significant differences when these data were subjected to statistical treatment.

Hair growth

Hair samples were clipped from the backs of rats on the 440th and 746th days of the experiment. The diameters of ten hairs from each rat were measured under the microscope with three readings at different places near the center of the hair. The diameters of these hairs are included in table 3. The hair of the female is always finer than that of the male even if the animals are maintained at the same body weight. At 460 days of age when group III animals weighed 140 gm. and those of group II weighed 100 gm., the hair of all retarded-growth animals was finer than that of the members of group I that were mature in size. At 746 days when group III had attained a mean weight of 170 gm. the hair had become as coarse as that of group I. The hair of group I had changed little if any. The hair of group II, the average weight of each member being 130 gm., had grown coarser but was still finer than that of the other groups.

When correlated with the body weight and age, these data indicate that hair grows coarser with both the increase in size and the increase in age of the individual. The hair changes may indicate that the rat is losing the qualities that characterize youth including the power to grow to a maximum size. It will be recalled from table 1 that our rats retarded for more than 700 days could not attain full adult size, while Osborne and Mendel claimed their rats could attain normal size after more than 500 days of retardation.

Organ weights

At the time of death the rats were dissected and the weights of the heart, liver, spleen and kidneys were recorded. The blood was pressed from the heart before weighing. Only these data for animals that lived to grow without restriction

are included in table 3. These are the same animals whose maximum weights are recorded in table 1. No animals are included if they died before the opportunity to grow was provided. The number of animals upon which these organ

TABLE 3

The effect of retarded growth upon the size of organs, hair and bones

	GROUP					
	I ♂	I ♀	II ♂	II ♀	III ♂	III ♀
Body weight in grams less organs, less gastro-intestinal tract[1]	214.5	148.6	133.1	103.6	120.5	110.6
Mean hair size in millimeters at 460 days	0.073	0.066	0.060	0.053	0.054	0.051
Mean hair size in millimeters at 746 days	0.071	0.063	0.064	0.059	0.070	0.062
Mean volume of femur in cubic centimeters	0.605	0.445	0.422	0.353	0.445	0.379
Mean weight of femur in grams	0.741	0.540	0.486	0.398	0.484	0.432
Density of femur	1.22	1.21	1.15	1.13	1.09	1.14
Mean length of femur in centimeters	3.85	3.48	3.43	3.26	3.54	3.36
Mean smallest diameter of femur in centimeters	0.35	0.32	0.33	0.31	0.34	0.32
Mean weight of liver in grams[2]	11.49 (11)	10.06 (18)	9.33 (7)	6.82 (13)	8.55 (9)	8.72 (10)
Mean weight of kidneys in grams	3.29	2.58	2.06	1.78	2.17	1.96
Mean weight of spleen in grams	0.82	0.81	0.47	0.47	0.75	0.75
Mean weight of heart in grams	2.04	1.81	1.57	1.16	1.55	1.21
Mean body length (nose to anus) in centimeters	21.9	19.8	18.4	18.0	18.1	17.5

[1] The organs include those listed as well as the lungs and genital organs.

[2] The numbers in parentheses give the number of animals used for organ weights.

weight data are based is shown by the figures in parentheses after the mean liver weight data. These numbers also refer to the oldest animals in each of the groups of table 2.

With the exception of group III, the livers of the males are larger than those of the females. In group III they are

about the same. The livers are smaller in the retarded groups than in those that matured rapidly, no. I. In spite of the fact that these are the livers of very old animals, they bear a close relation to the body weight at the time of death. These body weights, less the gastro-intestinal tracts and less the organs given in this table plus the lungs, are shown in the first line of table 3. Many of these animals were thin and emaciated at the time of death.

The kidneys of the males are consistently larger than those of the other sex. The kidneys do not correspond to the body weights at the time of death according to the tables of Donaldson ('24). On the other hand, these kidneys correspond very closely to the maximum weights attained by these various animals. If the maximum body weights of these rats of groups I ♂ to III ♀, respectively, are calculated from the kidney weights by the use of Donaldson's tables, one gets values of 420, 325, 240, 215, 268 and 235 gm., while from table 1 one can secure the mean maximum weight values for the same groups. These prove to be 439, 355, 262, 210, 267 and 227 gm., respectively. The similarity of these values is striking. Two explanations are apparent. One is that the kidneys of all groups were enlarged at the time of maximum weight and have decreased proportionately as the animal body wasted away before death. In the light of the findings of McLennan and Jackson ('33), however, it is more likely that the kidneys remained very close to their maximum weight even when the animals died in extreme old age. The shrinkage of the liver also corresponds to the findings of these authors. Due to the high protein content of our diets some enlargement of these kidneys might have been expected.

The spleens of the opposite sexes in the three groups are the same in weight. The spleens of group II are small, but no explanation for this size is available.

The hearts of all animals are much larger than even the maximum weights of the animals would indicate. These values exceed the highest given by Donaldson. This indicates that these hearts were considerably enlarged at the time of

death. This may be part of the pathology of old age or it may be due to some constituent of our diets. In our laboratory it has been observed that the hearts of guinea pigs can be enlarged in the course of a few months by feeding cod liver oil at levels lower than that employed in these diets. It cannot be stated at this time if this is the responsible agent in this case.

The hearts of the females are consistently lighter than those of the males. The hearts of the retarded groups are also lighter than those of group I. These organ data strengthen the thesis that the retarded animals failed to attain the body size of those that matured rapidly. Inasmuch as the male organs in the retarded groups are larger than the corresponding ones of the opposite sex, in most cases, this affords some indication that these organs may have shared in the retention of the superior growth potential that characterized the males of the retarded animals.

Bone growth

In order to study the growth of the bones in the three groups, all animals were preserved in formaldehyde. After the last animal had died, the femurs were removed. After these had dried in air the last traces of tissue were removed. The maximum length and the minimum diameter of each femur was measured.

Some of the femurs of the retarded groups proved to be very fragile. Some crumbled in the course of dissection. Part of these femurs from the retarded groups proved to be only thin cylinders of bone.

After the length and diameter were measured, each femur was weighed in air and then weighed suspended in water. The volume of each was then calculated. These measurements upon the femurs are included in table 4.

The bone measurements fit the general picture of the final size attained by the various groups. The bones of the males are consistently larger than those of the females in all groups. The bones of the retarded animals of the same sex are con-

siderably smaller than those of group I which grew to maturity rapidly. Inasmuch as the femurs of the males of the retarded groups are larger than those of the females there is some indication that the bones as well as the organs and the entire body shared in the final growth at the end of the long retardation. All measurements indicate that the femurs of group III were slightly larger than those of the same sex of group II. This suggests that some advantage in bone growth resulted from the initial gain in body weight which was allowed group III at the very beginning of the experiment.

As soon as the bones of groups II and III were weighed in water it was evident that they were less dense than those of group I. Some of the femurs of II and III floated. Some contained a small hole and immediately filled with water. These latter were discarded. In considering these data it will be observed that the bones from groups II and III were from extremely old animals while those from no. I were much younger.

The values for the density are the same for the opposite sexes, but the animals that grew to maturity early had bones of greater density. The capacity for bone growth may be lost earlier than that for other parts of the body.

Maintenance requirements of retarded-growth animals

The calories needed for maintaining the individuals of groups II and III at fixed levels of body weights were ascertained with accuracy for each. This was essential to maintain the body weights at fixed levels. The requirements at these various weight levels are summarized in table 4.

The females consistently require more calories for maintenance at the various weight levels.

The 't' values for these data were computed by the method of Livermore ('34). In group II the odds are 255:1 and in group III 9999:1 that the differences in calory requirements by the opposite sexes are significant.

Morris, Palmer and Kennedy ('33) found the female rat to be less efficient than the male in the utilization of food.

Part of this inefficiency of the female sex can be attributed to the slower growth rate. Part may be due to this higher maintenance requirement which exists when both sexes are forced to maintain the same body weight.

TABLE 4

Calory requirements for maintenance of the body weight at various levels

GROUP II			GROUP III		
Body weight level	Average calories per day per 100 gm. rat		Body weight level	Average calories per day per 100 gm. rat	
	♂	♀		♂	♀
gm.			gm.		
40	33.4±0.69	34.7±0.19	80	26.8±0.80	27.7±0.75
50	28.6±1.16	30.4±0.97	90	22.6±0.63	23.5±0.67
60	25.0±0.72	27.3±0.52	100	21.1±0.58	22.2±0.58
70	21.0±0.34	22.5±0.27	110	18.3±0.26	19.6±0.28
80	21.9±0.33	23.5±0.25	120	18.7±0.26	20.2±0.23
90	20.5±0.52	21.4±0.48	130	19.4±0.30	20.3±0.43
100	20.6±1.06	21.9±0.55	140	18.4±0.23	19.2±0.24
110	18.4±1.00	18.8±0.26	150	16.9±0.35	16.6±0.24
120	20.9±0.18	20.8±0.52	160	19.0±0.33	20.7±0.54
130	20.9±0.46	20.1±0.45	170	18.3±0.57	19.5±0.64

TABLE 5

Gross pathology at the time of death, in per cent of entire group[1]

GROUP NO.	LUNGS	KIDNEYS	GENITAL TRACT	TUMORS
	Per cent	*Per cent*	*Per cent*	*Per cent*
I ♂	78.0	14.0	...	14.0
I ♀	54.0	36.0	45.0	18.0
II ♂	58.0	16.0	8.0	8.0
II ♀	35.0	9.0	22.0	22.0
III ♂	85.0	50.0	...	7.0
III ♀	35.0	8.0	15.0	10.0

[1] This table is based on the number of animals in any one group showing definite types of infections on post mortem examination.

Pathology at the time of death

In the course of nearly 4 years in which this experiment was in progress many different pathological conditions were observed that are rarely seen in rat colonies. This was due to the maintenance of old animals in contrast to the usual

breeding colony for rats where individuals are usually discarded shortly after middle life.

The roughness of the fur coats of the old animals became apparent much earlier in those that matured rapidly. This is well illustrated in the photograph included in our preliminary report. As the experiment progressed many animals became blind. This happened so gradually that no quantitative data were secured, but a rough estimate would include at least half of the animals that lived beyond 2 years.

Old rats were frequently afflicted with diseases of the urinary tract. At times bloody urines were observed. In some cases individuals were treated with hexamethylenetetramine, but such treatment was usually futile. The old rat undoubtedly is a fertile experimental animal for the urologist.

In table 5 an attempt has been made to summarize some of the observations of the gross pathology at the time of death. The failure of the lungs is obvious in many cases, but this must have been secondary to many of the other failures that occurred in the old bodies.

SUMMARY

Rats were retarded in growth and not allowed to attain maturity until after periods of 766 and 911 days. The rat body still retains the power to grow at these extreme ages. After such periods of retardation the rat cannot attain a body size equal to that of an animal that grows to maturity younger. This conclusion is based upon the smaller size of the entire body, the weight of such organs as the heart, and the size of the bones represented by the femur. Even after these long periods of suppressed growth the male rat retains a growth potential greater than the female although the males of the retarded groups grow no larger than the normal females of this species. The hearts of all these animals dying in old age were larger than normal while the livers were smaller. The kidneys corresponded in weight at the time of death to the maximum weight attained by the body. The femurs of members of the retarded groups were less dense than those that matured normally.

In both retarded groups individuals of both sexes attained extreme ages beyond those of either sex that grew normally. The mean age of the males of both retarded groups was greatly increased in comparison with 'rapid growth' males while the mean age for the females was about the same in all three groups. The males of the retarded groups exceeded the females in age in contrast to the 'rapid-growth' group.

At a constant weight level in the course of retarded growth the female requires more calories for maintenance than the male. In the course of retarded growth, the diameter of the hair as well as the growth of the body reflects the retardation.

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VARIATIONS IN URINARY REDUCING SUBSTANCES OF TWO NORMAL DOGS MAINTAINED ON BREAD DIETS [1]

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TWO FIGURES

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Procedures which are employed to determine the gross availability of carbohydrates for animal nutrition fail to distinguish the more subtle changes which may occur in the carbohydrates of foods prepared by cooking, baking, canning, and other treatments. Regardless of conflicting interpretations[2] of the metabolic significance of sugar-like reducing substances in normal urine, further knowledge of the relations between diet and these normal reducing substances of urine should aid in the evaluation of the carbohydrates.

[1] This study was supported by a grant from Standard Brands, Inc.

[2] Benedict and Osterberg ('18), Benedict, Osterberg and Neuwirth ('18), Benedict and Osterberg ('23) and Neuwirth ('22) believe that there is a 'glycuresis' or excretion of sugar in normal urine intimately related to the ingestion of food. Folin and Berglund ('22) claim that the glycuresis represents not utilizable sugar but carbohydrate moieties from food rendered unassimilable by processes of cooking and baking. In this latter opinion, Greenwald, Gross and Samet ('25) concur. Recently West, Lange and Peterson ('32) have supported the original 'glycuresis' idea of Benedict with results that indicate a very small quantity of fermentable material (glucose†) is always present in urine even during fasting. This fasting excretion of fermentable material is denied by Harding and Selby ('33) and the present authors (Laug and Nash, '35) have cited some experiments which make it seem likely that a portion of the so-called fermentable material is formed from a precursor by hydrolysis during preparation of the urine filtrates with acid $HgSO_4$-$BaCO_3$.

We therefore report experiments in which we have examined total, fermentable, non-fermentable, and hydrolysable reducing substances in normal urines upon diets restricted to bread.

METHODS AND PROCEDURES

Breads were obtained from two local bakeries having a large distribution. (In the tables the source of the bread is indicated as 'K' and 'W'.) Because of variations in moisture content which would render comparisons from day to day difficult, the sliced breads were dried to constant weight, without toasting, in a specially constructed warm air drier (Laug, Garavelli and Nash, '34). An average loss of 35 per cent of the fresh weight occurred. The dried slices were then finely ground either whole, or, for some experiments, after the crust and white inside portions had been separated.

Analyses of the dried bread powders are given in table 1. Total carbohydrate was determined by Allihn's method after initial hydrolysis of starch by Taka-Diastase and subsequent mild hydrolysis by HCl (Olmsted, '20). The carbohydrate material amenable to Taka-Diastase hydrolysis was somewhat less in whole wheat and rye breads than in wheat bread. Free sugar in one sample of wheat bread amounted to 9 per cent.

Two hundred and fifty grams of the bread powder were moistened with water to make a thick mush and fed once daily to each of two female dogs of approximately equal weight (17 kilos). Dog I, a young police dog about a year old, gained weight slowly over the diet period of 3½ months. Dog II, a mongrel of bull dog type, maintained a steady weight throughout the diet period of 4 weeks. Except for the first few days, when the bread diet was instituted, the animals exhibited normal appetite and ate with great relish. No constipation developed, except over a 3-day regime during which one dog received only the white inside portion of the bread. A mildly laxative action of the crust was clearly exhibited. Both animals were alert and showed all signs of well-being.

Urine collections were made by catheter, with adequate washing of the bladder at intervals of 2 to 4 hours throughout a 12-hour period after feeding. Moderate pains were taken to maintain sterile technic, at no time was there ever any evidence of bladder infection. All urines were acid to litmus save those collected in the first post feeding period, when a slight alkaline tide usually occurred. Urines were preserved with toluene and kept on ice.

Urine filtrates were prepared by the acid $HgSO_4$-$BaCO_3$ procedure of West and Peterson ('32). Reducing materials

TABLE 1

Analyses of the dried bread used in the diet experiments

KIND OF BREAD	MOISTURE CONTENT	PROTEIN[1]	TOTAL CARBOHYDRATE AS GLUCOSE
	per cent	*per cent*	*per cent*
K. Wheat	0.8	15.75	79.0
K. Wheat (crust)	1.6	15.13	81.8[2]
K. Wheat (white inside)	1.8	15.25	82.8[2]
W. Wheat	0.5	14.25	78.1
W. Rye	1.2	13.50	76.8
W. Whole wheat	1.3	15.20	72.7

[1] Protein = total nitrogen × conversion factor 6.25.

[2] By direct mineral hydrolysis of starch, instead of by Taka-Diastase.

in the filtrates were determined with the sensitive Shaffer-Hartmann reagent no. 50, containing 1 gm. of KI per liter (Shaffer and Somogyi, '33). Fermentation technic was that of Somogyi ('27). Hydrolysis of filtrates was accomplished by making them approximately 1 N with H_2SO_4 and heating on the water bath for 2½ hours. After cooling the hydrolysate and diluting with water to 50 ml. volume, the mixture was neutralized with dry $BaCO_3$.

EXPERIMENTAL

It has been known for some time that ingestion of carbohydrates degenerated by heat produces a large increase in urinary reducing substances. Particularly is this true of

such food products as caramelized sugars, Karo syrup, Grape Nuts, and even ordinary toast. Folin and Berglund ('22) have postulated that a considerable part of the reducing materials excreted in urine does not reflect the course of events in carbohydrate metabolism but represents merely the escape of carbohydrates rendered unassimilable by processes of cooking and baking. Figure 1 compares the results of feeding the white inside and crust parts of bread in daily 250 gm. portions. A considerable rise in the total reducing values after crust feeding reflects chiefly the increase in the non-fermentable fraction. The slight rise in the fermentable values may be due to hydrolysis during preparation of the urine filtrates by the strongly acid mercuric sulphate reagent (Laug and Nash, '35). Pari passu with the increase in non-fermentable reducing substances is the well-marked rise in total nitrogen. It is particularly interesting to note that the output of reducing and nitrogenous substances is exceedingly slow. With either type of food, the 4-hour periods 8 hours after feeding still show a maximal excretion even of the fermentable fraction alleged to be glucose. Höst ('23) regards such time relations as evidence that excretion of reducing material does not represent normal carbohydrate metabolism; he has shown that the rise of such materials in the blood is prompt and precedes by several hours the rise in the urine. Likewise, Folin and Berglund ('22) say: "Since the sugar of normal urine is quite independent of the level of the blood sugar, it must be considered exceedingly doubtful whether the sugar of normal urine is glucose, quite independently of whether it does or does not ferment." Wang and Felsher ('24) found in the urine considerable increases in reducing materials after feeding discolored commercial glucose, whereas alcoholic extracts of the brown coloring material containing presumably denatured carbohydrates, produced no rise in blood sugar. It may be that the kidneys effect a relatively high concentration of non-fermentable (non-assimilable?) substances from the blood (West, Lange and Peterson, '32). It is, of course, also possible that the digestive function of the dog may be very slow or deficient in respect to

starch. But we have observed (Laug, Garavelli and Nash, '34) that in the phlorhizinized dog a delayed excretion of sugar is not evident. In this type of renal diabetic there is a sharp rise in excretion 2 to 3 hours after feeding bread, followed by a rapid return to a low level. The question may be raised whether in the phlorhizinized animal there is an enhanced process of assimilation of sugar from starches to meet the urgent need of the body, or whether in the normal animal

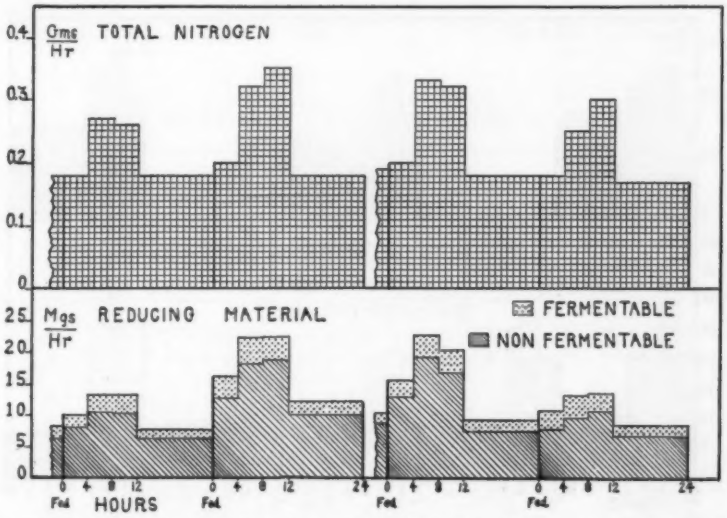

Fig. 1 Excretion of reducing material and total nitrogen in the urine of dog I after feeding white inside (first and last periods) and crust (second and third periods) of wheat bread.

the slow excretion is a reflection of unknown factors in carbohydrate metabolism. We are inclined to the latter view.

In summarizing seven 24-hour periods (four of which appear in fig. 1), it was found that on 4 days of white inside feeding, the averages of reducing materials excreted were: Total, 235.4 mg. (range, 211–246); non-fermentable, 182.9 mg. (range, 166–192) and fermentable, 52.3 mg. (range 45–59). During 3 days of crust feeding the averages were: Total,

364.2 mg. (range, 346–386); non-fermentable, 301.5 mg. (range, 286–318); and fermentable, 62.7 mg. (range, 60–68). Of the average increase in total, (364.2 minus 235.4 equals 128.8 mg.), 92 per cent was accounted for by the increase in the non-fermentable and only 8 per cent by the fermentable fraction.

Figure 2 describes the excretion of reducing substances for 5 successive days during which wheat bread, rye bread, and

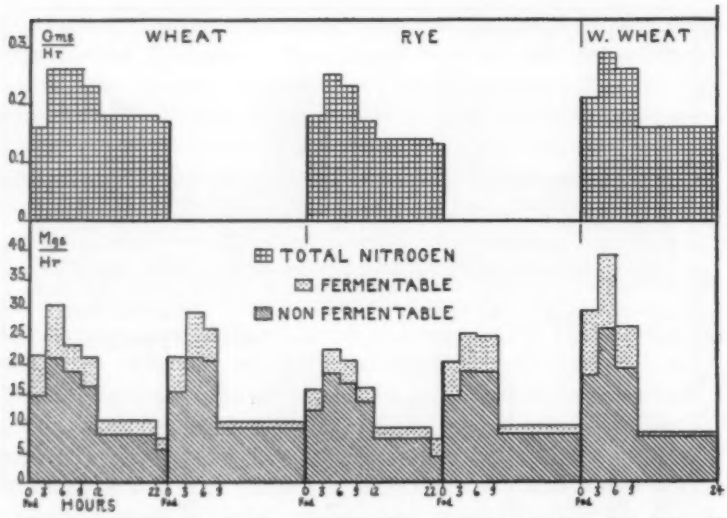

Fig. 2 Excretion of reducing material and total nitrogen in the urine of dog II after feeding wheat, rye, and whole wheat bread.

whole wheat bread were fed in rotation. The results indicate that the excretion of non-fermentable and fermentable materials is lowest after feeding rye bread, intermediate after wheat bread, and highest after whole wheat bread. While fermentable materials are always present, even in 2-hour urines preceding feeding, the value falls almost to zero during the night after the whole wheat bread meal. Gross analyses of breads (vide table 1) are unable to define such distinctions, except to indicate that whole wheat and rye breads contain somewhat less starch and more fibrous substance.

TABLE 2

The excretion of reducing materials (before and after hydrolysis) in the urine of dog I after feeding three different bread preparations

DATE 1934	URINE COLLECTION	URINE VOLUME	REACTION TO LITMUS	TOTAL N	REDUCING SUBSTANCES			REDUCING SUBSTANCES PRODUCED BY HYDROLYSIS		NON-FERMENTABLE REDUCING SUBSTANCES	
					Total	Fermentable		Total	Fermentable	Before hydrolysis	After hydrolysis
		cc.		Gm. / hour	Mg. / hour	Mg. / hour	Per cent of total	Mg. / hour	Mg. / hour	Mg. / hour	Mg. / hour
4/5	10.00 A.M.–12.00 M.	10	S. alk.	0.13	7.9	1.8	22.3	3.8	2.9	6.1	7.1
fed 250 gm.	12.00 M. – 3.06 P.M.	42	S. alk.	0.21	11.5	1.6	13.5	7.0	6.7	9.9	10.2
wheat bread	3.06 P.M.– 7.30 P.M.	110	Acid	0.41	13.0	2.5	19.7	5.8	6.4[1]	10.5	9.8
(K) at	7.30 P.M.– 8.00 A.M.	245	Acid	0.19	8.2	1.6	19.9	3.9	4.0	6.6	6.7
10.00 A.M.	8.00 A.M.–10.00 A.M.	6	Acid	0.10	4.0	0.3	8.4	2.0	1.7	3.5	3.9
	Totals for 24 hours			5.275	219.1	39.9	18.2	106.9	108.1 (101% of total)	178.8	178.3
4/6	10.00 A.M.– 1.00 P.M.	25	S. alk.	0.17	7.5	1.7	22.3	3.6	2.8	5.9	7.7
fed 250 gm.	1.00 P.M.– 5.00 P.M.	60	S. acid	0.24	11.0	2.1	19.1	8.3	7.2	8.9	10.1
wheat bread	5.00 P.M.– 9.06 P.M.	70	Acid	0.26	12.8	2.7	21.0	8.8	7.6	10.1	11.3
(K) at	9.06 P.M.–10.00 A.M.	310	Acid	0.22	8.1	1.2	14.9	3.3	3.2	6.9	7.1
10.00 A.M.											
	Totals for 24 hours			5.354	223.1	40.0	17.9	123.6	109.0 (88.2% of total)	183.2	197.7
4/7	10.00 A.M.–12.00 M.	8	Acid	0.14	8.6	1.3	15.5	5.8	5.3	7.2	6.6
fed 250 gm.	12.00 M. – 3.00 P.M.	30	Acid	0.22	15.8	2.9	18.1	7.2	7.6	13.0	12.5
crust from	3.00 P.M.– 7.30 P.M.	95	Acid	0.30	20.2	4.2	20.7	7.7	8.6	16.0	15.1
wheat bread	7.30 P.M.–10.10 A.M.	312	Acid	0.19	11.0	1.9	17.7	5.0	4.6	9.1	9.6
(K) at											
10.00 A.M.											
	Totals for 24 hours			5.53	316.9	57.8	18.2	141.3	138.9 (98.3% of total)	259.2	261.6
4/16	9.00 A.M.–11.00 A.M.	30	S. alk.	0.18	9.0	2.2	24.7	4.6	4.3	6.8	8.4
fed 370 gm.	11.00 A.M.– 2.00 P.M.	45	Alk.	0.26	11.4	2.9	25.2	6.6	5.7	8.5	9.5
fresh wheat	2.00 P.M.– 5.00 P.M.	114	Acid	0.31	11.5	3.1	26.9	8.2	6.7	8.4	9.8
(K) bread ⇔	5.00 P.M.– 8.00 P.M.	87	Acid	0.29	11.3	2.9	25.3	7.4	6.0	8.5	9.8
250 gm. dried	8.00 P.M.– 8.00 A.M.	435	Acid	0.18	5.9	1.2	21.0	2.8	2.2	4.7	5.5
at 9.00 A.M.											
	Totals for 23½ hours			5.198	198.8	47.5	24.0	111.0	87.8 (79.2% of total)	151.3	173.5

[1] In cases where the fermentable appears higher than the total fraction after hydrolysis, it indicates that some of the non-fermentable material has contributed, by being itself slightly hydrolysed.

Table 2 presents in addition to the total and fermentable reducing substances in urine, data on the reducing substances produced by hydrolysis, after diets of whole bread, fresh and dried, and crust. Before discussing these it should be noted that the presence of crust (about 19 per cent) in whole bread appears to account for an increase in the non-fermentable fraction over the period when the diet was crust free (white inside). Compare 219.1 and 223.1 mg. after whole bread, with the average 182.9 mg. on white inside. Also, while toasting has never occurred in our process of drying bread, it is interesting to note that when fresh bread in amount equivalent to dry bread is fed, the distribution of fermentable and non-fermentable reducing substances in the urine indicated an increase in the former fraction and a decrease in the latter.

The total hydrolysable fraction follows rather closely total reducing values. After feeding, the two rise and reach their highest levels at approximately the same time. The hydrolysable value never falls to zero, even 22 hours after feeding. The fermentable and non-fermentable products of hydrolysis are distributed, however, quite differently from the non-hydrolysed. In agreement with observations of West, Lange and Peterson ('32) and Patterson ('26) practically 100 per cent of the hydrolysed material is fermentable. The hydrolysed substance is not produced at the expense of the original non-fermentable reducing fraction. The data of table 2 show no significant change in value of the non-fermentable fraction after hydrolysis.

Edwards and Everett ('34) in their experiments on toast and Grape Nut feeding to man find no increase in the hydrolysable fraction but in most cases observe destruction of reducing material by hydrolysis. If the substances of bread crust are the same as those produced by toasting our results are not in accord with those of Edwards and Everett. Indeed, we find after crust feeding a slightly larger hydrolysable fraction than after whole bread feeding. Greenwald, Gross, and McGuire ('27), upon feeding heated sugar found no increase in non-fermentable reducing substances in the urine of man,

TABLE 3

The excretion of reducing materials (before and after hydrolysis) in the urines of dogs I and II after feeding wheat and whole wheat bread

DATE 1934	URINE COLLECTION	URINE VOLUME	REACTION TO LITMUS	TOTAL N	REDUCING SUBSTANCES			REDUCING SUBSTANCES PRODUCED BY HYDROLYSIS		NON-FERMENTABLE REDUCING SUBSTANCES	
					Total	Fermentable		Total	Fermentable	Before hydrolysis	After hydrolysis
		cc.		*Gm./hour*	*Mg./hour*	*Mg./hour*	*Per cent of total*	*Mg./hour*	*Mg./hour*	*Mg./hour*	*Mg./hour*
Dog II	10.00 A.M.– 1.00 P.M.	47	Neut.	0.24	18.3	4.2	25.2	3.1	4.3	14.1	12.9
5/28	1.00 P.M.– 4.00 P.M.	125	Sl. acid	0.31	28.7	8.6	30.1	5.4	6.5	20.1	19.0
fed 250 gm.	4.00 P.M.– 7.00 P.M.	158	Acid	0.30	24.3	6.8	28.5	0.8	4.0	17.4	14.3
wheat bread	7.00 P.M.–10.06 P.M.	100	Acid	0.23	16.4	3.2	20.0	4.2	5.8	13.1	11.5
(W) at	10.06 P.M.– 8.00 A.M.	267	Acid	0.14	6.7	0.67	10.1	2.1	1.6	6.1	6.6
10.10 A.M.	8.00 A.M.–10.00 A.M.	30	Sl. acid	0.12	4.9	0.65	13.4	2.7	1.4	4.2	5.4
	Totals for 24 hours			4.94	341.1	77.1	22.6	66.8	80.3 (112% of total)	264.0	250.5
Dog II	10.00 A.M.– 1.00 P.M.	40	Neut.	0.21	27.9	11.2	40.0	— 1.3	— 0.4	11.8	15.7
5/29	1.00 P.M.– 4.00 P.M.	110	Acid	0.36	41.2	13.3	32.5	0.1	1.7	27.7	26.0
fed 250 gm.	4.00 P.M.– 7.03 P.M.	117	Acid	0.32	26.7	6.5	24.2	1.0	0.23	20.3	21.0
whole wheat	7.03 P.M.–10.00 P.M.	75	Acid	0.22	13.8	4.4	32.3	4.0	0.37	9.5	13.1
bread (W) at	10.00 P.M.– 8.00 A.M.	192	Acid	0.16	6.6	0.12	1.8	1.8	0.35	6.4	7.9
10.10 A.M.	8.00 A.M.–10.00 A.M.	28	Sl. acid	0.14	5.2	0.0	0.0	3.4	0.0	7.3	10.0
	Totals for 24 hours			5.18	405.0	107.3	26.5	36.1	9.7 (26.9% of total)	301.8	326.8
Dog. I	10.15 A.M.– 1.15 P.M.	35	Neut.	0.22	14.7	5.1	35.0	3.6	5.0	9.5	8.2
5/28	1.15 P.M.– 4.15 P.M.	35	Acid	0.27	23.7	7.4	31.0	4.8	7.0	16.3	14.1
fed 250 gm.	4.15 P.M.– 7.15 P.M.	57	Acid	0.26	24.3	7.2	29.5	6.2	5.8	17.1	17.8
wheat bread	7.15 P.M.–10.21 P.M.	52	Acid	0.24	19.2	5.3	27.4	7.7	6.4	14.0	15.2
(W) at	10.21 P.M.– 8.15 A.M.	328	Acid	0.18	10.7	2.3	21.6	4.5	2.7	8.5	10.2
10.20 A.M.	8.15 A.M.–10.15 A.M.	78	Acid	0.13	5.8	1.5	25.9	3.8	1.0	4.3	7.1
	Totals for 24 hours			5.09	365.3	101.4	27.8	120.4	102.4 (85.2% of total)	263.9	282.7
Dog I	10.15 A.M.– 1.15 P.M.	32	Acid	0.17	24.4	11.0	44.8	0.0	0.53	13.5	12.9
5/29	1.15 P.M.– 4.15 P.M.	87	Acid	0.29	37.9	12.0	31.7	1.2	3.8	25.9	23.3
fed 250 gm.	4.15 P.M.– 7.18 P.M.	82	Acid	0.30	31.4	13.7	43.4	3.8	0.52	17.8	21.0
whole wheat	7.18 P.M.–10.15 P.M.	55	Acid	0.25	21.9	5.1	23.1	4.6	3.8	16.9	17.8
bread (W) at	10.15 P.M.– 8.15 A.M.	183	Acid	0.16	10.4	1.6	15.1	2.2	— 0.37	8.9	11.4
10.20 A.M.	8.15 A.M.–10.15 A.M.	72	Acid	0.15	6.0	0.15	2.5	2.5	0.0	5.9	8.5
	Totals for 24 hours			4.95	464.1	141.7	30.5	55.6	21.8 (39.4% of total)	321.0	326.2

but a considerable increase in the dog with a rise in the hydrolysable fraction. It is possible that species differences in excretion and nature of urinary reducing substances may be more important than have been recognized.

The major interest in the data of table 3 is the contrast in results obtained upon diets of wheat bread and whole wheat bread, respectively. The total reducing urinary output is considerably higher upon the whole wheat bread feeding. Of the total increase, 47 per cent (dog II) and 41 per cent (dog I) occurs in the fermentable fraction. This behavior is quite different from that obtained after crust feeding, where the chief rise is in the non-fermentable fraction. If the increase in the non-fermentable fraction after crust feeding is any indication of the presence in the blood of non-utilizable sugar-like substances, the urine picture after whole wheat bread feeding might be expected to be qualitatively similar. In both dogs the total hydrolysable material is decreased about 50 per cent upon the whole wheat bread diet. During several periods the hydrolysable fraction falls practically to zero, and in one case there is actual loss of reducing value associated with hydrolysis. The fermentable fraction of the total hydrolysable material is considerably smaller. This is also apparent from the increase in the non-fermentable values after hydrolysis. In terms of an hypothesis proposed elsewhere (Laug and Nash, '35), these findings indicate that hydrolysis was not completed in all cases, with the resulting accumulation of the first stage of hydrolysis material which is not fermentable. An unexplained effect is noted in the behavior of the fermentable fraction of the night and following morning samples of urine after whole wheat bread feeding; this becomes vanishingly small or disappears entirely.

Although the analyses of breads from the two different bakeries show essentially agreeing values for total carbohydrates, the feed-experiments on dog I which received these two kinds of bread (compare tables 2 and 3) indicate differences in excretion of reducing materials. The level of excretion of total, non-fermentable, and fermentable after bread W

was higher than with bread K. But the distribution of fermentable and non-fermentable substances was different. With bread W the fermentable fraction was higher (27 per cent as compared with 18 per cent). This difference is probably accounted for in the methods of baking. No significant differences in the total hydrolysable fractions are apparent.

After feeding, the hourly rate of excretion in dog II reached a peak higher and usually more rapidly than in dog I; while dog I, excreting more slowly, put out a larger amount in the whole period.

SUMMARY

A study has been made of the excretion of reducing substances in the urine of two normal dogs maintained on bread diets.

Significant variations in the total, fermentable, non-fermentable, and hydrolysable reducing substances can be correlated with the particular kind of bread diet.

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THE INFLUENCE OF CHRONIC FLUORINE TOXICOSIS IN LAYING HENS UPON THE FLUORINE CONTENT OF THE EGG AND ITS RELATION TO THE LIPOID CONTENT OF THE EGG YOLK [1]

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In a previous study (Phillips, Hart and Bohstedt, '34) it has been shown that fluorine in the ration of dairy cows does not influence the nutritional qualities of milk. In so far as we could determine chemically and biologically, there was no significant increase in fluorine content of the milks produced. The chicken has been shown to be more resistant to fluorine toxicosis than other species (Halpin and Lamb, '32). This knowledge is very apt to lead to over confidence in the use of fluorine containing mineral supplements—since it develops a feeling of safety with respect to poultry products for human consumption. It was with this problem in mind that a study of the fluorine content of the eggs from chronically poisoned laying hens was undertaken.

Most of the studies reported in the literature concerning the effects of fluorine in the nutrition of poultry deal with the levels of tolerance and the physiological responses to fluorine ingestion. Puresz et al. ('34) reported the appearance of fluorine in the egg following intravenous injections of 30 micrograms of NaF every fifth day. The fluorine content was reported to have reached the maximum value of

[1] Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

46.3 micrograms per cent. This gradually receded upon the discontinuance of the injections. No fluorine was found in the normal egg although the method was reported to be sensitive to fluorine in quantities of 25 to 400 micrograms. Continued injection of F retarded egg production and there was a gradual loss of egg weight. The data reported covered only two birds and the period of time was relatively short since it covered only 2½ months.

EXPERIMENTAL

The animals used in these studies were birds grown on rations designed to study the influence of fluorine. They had almost finished their second laying year at the time the eggs were collected for study. They had been on their experimental rations and subjected to the chronic influence of fluorine for approximately 28 months.

The rations used were similar to those previously used and described elsewhere (Halpin and Lamb, '32). The basal ration was composed of yellow corn 65 parts, wheat middlings 16 parts, dried skim milk 18 parts, and salt 1 part. Lot I received this ration unsupplemented by mineral additions. Lot II received 95 parts of the basal ration with 2 parts of ground limestone and 3 parts of steamed bone-meal as a mineral supplement. The rations of lots III, IV, and V were identical with that of lot II except 1 per cent, 2 per cent, and 3 per cent of raw rock phosphate was substituted, respectively, for like quantities of steamed bone meal. Except for lot I, the rations contained approximately 1.98 per cent Ca and 0.91 per cent P, thus providing a calcium: phosphorus ratio of about 2: 1.

The eggs were collected during the months of July and August. They were stored until fluorine determinations could be made. Three to 4 dozen eggs were obtained for each lot. A batch of twelve eggs was used for each set of determinations made. In preparation for analysis twelve eggs from each respective lot were washed in distilled water, then hard boiled and separated into three portions, shell, yolk, and

white. These were dried and quadruplicate samples were taken for analyses. Duplicate samples were run without added fluorine and to each of the other two samples 0.01 mg. of F was added. The samples were ashed repeatedly with Na_2CO_3 at low temperature until a white ash was obtained. The ash was then analyzed for F by the method of Willard and Winter ('33) using sodium alizarin sulphonate as the indicator.

RESULTS

The first analyses showed that little or no fluorine was present in the egg shell proper, but the whole egg from the lots receiving rock phosphate contained increased quantities of fluorine. A separation of the egg into the yolk and white indicated that traces of F only were present in the white. With this information at hand effort was concentrated upon the egg yolk. An attempt was made to determine with what constituents of the egg yolk the F was carried or combined.

The yolks of twelve eggs were continuously extracted by ether for 24 hours to obtain the ether soluble substances in an attempt to divide the yolk into three fractions, true fat, lipoid substances, and non-fat materials. It was found that ether extraction did not completely remove the fatty constituents. However, if ethyl alcohol was substituted for the ether and the extraction continued for 48 hours practically complete removal of the fatty material was accomplished. The alcohol from the alcohol extract was then removed by drying on a sand bath. The alcohol residue was next extracted repeatedly with anhydrous acetone until all acetone soluble products were removed. The acetone insoluble residue was next extracted with ether. Thus the yolk was roughly partitioned into three portions: a non-fat or alcohol insoluble residue, 31 to 38 per cent; a fat or an acetone soluble fraction, 42 to 48 per cent, and the acetone residue soluble in ether or the complex lipoids, 15 to 25 per cent. Each fraction was then analyzed for its fluorine content. The results are summarized in table 1.

Examination of table 1 indicates clearly that the inclusion of 0.035 to 0.105 per cent F in the ration in the form of rock phosphate causes an increase in the quantity of fluorine occurring in the egg yolk. Further, the fluorine seems almost completely to accompany the lipoid fraction of the yolk.

TABLE 1

The fluorine content of various constituents of the egg as affected by chronic F toxicosis in laying hens (milligrams of F per 100 gm.)

		WHITE	YOLK	YOLK-ACETONE RESIDUE (LIPOID FRACTION)	YOLK-ACETONE SOLUBLE FRACTION (FAT)	YOLK-ALCOHOL INSOLUBLE RESIDUE (NON-FAT)
Lot I no mineral	Average of samples without added F	0.02	0.09	....	None	None
	Average of samples calculated after deducting added F	0.03	0.08	....	None	None
Lot II limestone and bone meal	Average of samples without added F	0.02	0.12	0.17	None	None
	Average of samples calculated after deducting added F	....	0.12	0.16	None	None
Lot III 1 per cent rock phosphate (0.035 per cent F)	Average of samples without added F	0.02	0.18	0.27	Trace	None
	Average of samples calculated after deducting added F	0.01	0.20	0.29	Trace	None
Lot IV 2 per cent rock phosphate (0.07 per cent F)	Average of samples without added F	0.02	0.33	0.69	Trace	None
	Average of samples calculated after deducting added F	0.01	0.27	0.65	Trace	None
Lot V 3 per cent rock phosphate (0.105 per cent F)	Average of samples without added F	0.03	0.30	1.14	0.06	None
	Average of samples calculated after deducting added F	0.05	0.32	1.14	0.04	None

Traces of fluorine occurred in the fats and particularly at the 0.105 per cent level of fluorine feeding. This may be due to incomplete separation of the fats and lipoids. A relatively small amount of fluorine was found in the egg white. This is

believed to be the result of incomplete separation of the white and yolk after boiling and the values are therefore due in part at least to the lipoids of the yolk.

No change in egg size was noted in the course of these experiments. It seems that egg size is a genetic factor since severe fluorine toxicosis completely inhibited egg production rather than affected the size of the egg.

DISCUSSION

These results indicate that fluorine occurs in the whole egg yolk. Since the acetone insoluble fraction was approximately 20 per cent, it would be expected that the lipoid portion would contain four to six times more fluorine than in the yolk itself. We are unable to account for this discrepancy. It is possible that the acetone extraction of the fatty materials was not complete for this would tend to reduce the lipoid portion in the samples because of the true fat contamination, or that some of the fluorine was lost in the treatment of the egg yolks. The latter does not seem likely. However, for the purposes of this study, the results are sufficiently clear to demonstrate the accumulation of fluorine in the egg primarily in the yolk, and that the fatty constituents of the egg yolk contains nearly all of the fluorine. Further, the evidence distinctly indicates that the fluorine is combined with the acetone insoluble fraction, or the lipoids of the egg yolk.

It is interesting to note that the evidence presented here indicates another halogen-lipoid combination in biological material, a fact which was recently emphasized by Peters and Man ('34).

It is desired to point out that the amounts of fluorine reported here may be of concern to the public health. On the basis of these results 3 to 5 dozen eggs from hens fed 3 per cent of rock phosphate would furnish about 1 mg. of fluorine. Recently Smith ('35) has shown that it requires only 1 mg. of F per liter of drinking water to cause the mottling of teeth. While the source of danger of fluorine toxicity from the egg is not comparable to sources in drinking water, the

universal use of eggs in the human diet, and especially for growing children, suggests the need of caution in the widespread use of fluorine containing mineral supplements in poultry feeding.

SUMMARY AND CONCLUSIONS

The addition of fluorine in the form of rock phosphate to the rations of laying hens results in a distinct and measurable increase in the fluorine content of the eggs. The fluorine accompanies the constituents of the egg yolk. It is completely separated from the non-fat portion of the yolk by extraction of the fatty constituents with ether or ethyl alcohol. It appears that the fluorine wholly or in large part at least, remains with the acetone-insoluble portion of the fat-like substances of the egg yolk. This suggests that fluorine is deposited in the egg in combination with the complex lipoids of the yolk.

It appears that fluorine is present in minute quantities in the normal egg. Increased fluorine ingestion did not reduce the size of the eggs in this series of experiments. The possibility of harmful effects arising from eggs from fluorine fed hens is discussed.

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EDITORIAL REVIEW [1]

THE CHIEF SULFUR COMPOUNDS IN NUTRITION

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As an introduction to this review, a brief summary of the occurrence and chemistry of the sulfur compounds to be discussed and of the methods used in their study may be of value. Such a summary is presented in table 1 and will be commented upon briefly. Since the author has previously reviewed the earlier studies on the economy of sulfur ('24, '32, '33), the literature citations include for the most part only the more recent investigations.

Most important of the sulfur compounds of biological importance are the proteins which vary widely in their content of sulfur. On the one hand, we have the sulfur-poor proteins, as, for example, gelatin with a sulfur content of approximately 0.2 per cent, and many vegetable proteins, and on the other, the proteins of the epidermal tissues of the higher forms, the keratins, in some of which, as in human hair, the percentage of sulfur is more than 5 per cent.

In 1899, Mörner showed that this protein sulfur existed, in part at least, as the amino acid, cystine, discovered in 1810 by Wollaston as a constituent of a rare type of urinary calculus. It was soon recognized, however, that in a large number of proteins, even when the inadequacy of the existing analytical methods for the determinations of cystine was

[1] A revision of a paper presented before the Section on Medical Sciences of the American Association for the Advancement of Science as a part of a symposium on the "Chemistry and Metabolism of the Sulfur-containing Compounds of the body." Pittsburgh, December 28, 1934.

taken into consideration, the sulfur must also be present in some form other than cystine. In 1923, Mueller isolated a new type of sulfur-containing amino acid from protein hydrolysates, the structure of which was proven and the synthesis accomplished by Barger and Coyne in 1928. To the new sulfur-containing constituent of the protein molecule, the name methionine was given. The presence of methionine in

TABLE 1

Sulfur compounds in the living organism

A. Naturally occurring organic sulfur compounds containing nitrogen.
  1. Proteins (0.2 to 5.0 per cent S).
  2. Known constituents of the protein molecule—The sulfur-containing amino acids.
    a. Cystine—Wollaston—1810—calculi—Mörner—1899—protein.
    b. Methionine—Mueller—1923—Barger and Coyne—1928.
  3. Probable derivatives of protein or of the above amino acids.
    a. Taurine (taurocholic acid).
    b. Cysteine.
    c. Ergothioneine.
    d. Glutathione (reduced and oxidized).
    e. Insulin.
    f. Mercapturic acids.

B. Compounds related to or derivatives of naturally occurring sulfur compounds (the biological significance of these remains to be proven).
  1. Homologues of cystine or cysteine.
    a. Homocystine (homocysteine).
    b. Pentocystine (pentocysteine).
  2. Homologues of methionine.
    a. Homomethionine.
  3. Thiolhistidine.
  4. Products of partial oxidation of cystine and cysteine.
    a. Cysteic acid.
    b. 'Suboxidation products' of cystine and cysteine (Toennies-Hammett).

practically all types of proteins has now been demonstrated although the quantitative data are inadequate (Baernstein, '32 b). In some proteins, however, notably in casein, methionine is present in amount far in excess of the amount of cystine. Despite much experimental study, the isolation of other sulfur-containing amino acids from protein has not been accomplished.

Numerous other sulfur compounds of great biological interest are known to occur in the animal world, many of which appear to be derivatives of protein or of the amino acids present in the protein molecule. Of these, taurine, isolated as a hydrolysis product of the bile acids by Gmelin, has been known for more than a century. Friedmann postulated the formation of taurine in vitro by oxidation of cystine to cysteic acid and decarboxylation of the latter but recent workers have found difficulty in effecting the second reaction of this synthesis, nor is the derivation of taurine from cystine in vivo definitely established.

Cysteine may be readily formed from cystine by reduction ($RS — SR \rightleftarrows 2\,RSH$). In view of the interest in the function of the sulfhydryl group and of the discovery of glutathione, of which cysteine is a constituent, it is important to determine whether cysteine exists as such in the protein molecule or as its oxidation product, the disulfide, cysteine. Because of the ready oxidation of cysteine, its isolation, if present in the products of protein hydrolysis, presents difficulties which have as yet not been mastered and the occurrence of cysteine as a primary unit of protein structure is problematical.

Ergothioneine, isolated by Tanret from ergot in 1909 and identified as a constituent of blood in 1927, is one of the two betaines known to occur in the blood and tissues of the mammalian organism. Its origin from a sulfhydryl derivative of histidine, thiolhistidine, has been suggested. Thiolhistidine has recently been synthesized but attempts to prepare its methylated derivative have not been equally successful. The biological significance of ergothioneine is not known. Its presence in the blood, however, contributes to errors in those quantitative determinations which involve reductions, since its sulfhydryl group reacts similarly to that of glutathione.

The isolation and characterization of glutathione by Hopkins in 1921 is usually considered one of the important biochemical discoveries of recent years. Although the structure proposed was later shown to be incorrect by Kendall and by Hopkins himself, the discovery has influenced im-

portantly the recent studies of biological oxidations and reductions. Many of the investigations concerning its distribution in tissues, and the attempts to correlate distribution and function are invalidated by the inadequate and non-specific methods used for its determination. To cite one example only, the ubiquitous occurrence of ascorbic acid, which reacts similarly to glutathione in the iodine titration method most commonly used for the estimation of glutathione, has robbed much of the early work of its significance. A recent writer (Gibson, '34) has even doubted the generally accepted structure for glutathione and has minimized its importance biologically. "Nine years ago glutathione had been isolated and 'synthesized.' Today its constitution is far from decided and it seems that the purer it is obtained, the less does it behave in vitro as its biological significance would make us expect."

An acceptable synthesis of glutathione is urgently needed to establish its structure and a specific method for its determination will make possible significant advances in our knowledge of the distribution, origin, and function of this tripeptide.

With the successful crystallization of insulin in 1926 by Abel and his co-workers, the more exact knowledge of its chemistry has been made possible. That it is a protein or a derivative of a protein, and that for its proper functioning, the sulfur-containing component, probably cystine, is essential, seems definitely established by the work of Jensen, du Vigneaud and others (Jensen and Evans, '34).

The detoxication of certain benzene derivatives, notably the monohalogen substitution products, by the animal organism results in the formation of one of the most interesting and complex sulfur-containing compounds of which we have knowledge, bromophenylmercapturic acid. The synthesis of the mercapturic acids involves the reduction of cystine to cysteine, the acetylation of the amino group and the union of the benzene nucleus with the sulfhydryl group of cysteine. The effectiveness of this detoxication seems to be dependent largely upon the available supply of cystine (White and Lewis, '32).

In addition to these compounds of sulfur known to exist and function in the living organism, certain others, chemically related to these, have been prepared and studied in their biological relationships in the belief that information as to the metabolism of the naturally occurring sulfur compounds might result. Of this group, the homologues of cysteine (or cystine) and methionine are of greatest interest. Butz and du Vigneaud ('32) observed that when methionine was heated with sulfuric acid for some hours, demethylation occurred and the next higher homologue of cystine, which they designated as homocystine, could be isolated. The next higher member of this homologous series, pentocystine, and the next higher homologue of methionine, homomethionine, which bears the same relation to pentocystine as methionine to homocystine, have recently been synthesized (du Vigneaud, Dyer, Jones and Patterson, '34).

As already mentioned, thiolhistidine, the probable precursor of ergothioneine and important in that a sulfhydryl

group is here attached to a ring structure, has been synthesized. Although the attempts to demonstrate the presence of this amino acid in the protein molecule are suggestive, definite proof of its existence as a building stone of proteins is still lacking.

It is known that oxidation of cystine by such agents as hydrogen peroxide or nitrous acid gives rise to sulfuric acid. Since in the animal body, the oxidation of cystine also leads to the formation of sulfuric acid, the study of the in vitro oxidation of cystine is important. It has long been known that the oxidation of cystine to the sulfonic acid, cysteic acid, occurs readily but the resistance of this substance to further oxidation makes it improbable that the biological oxidation of cystine to sulfates follows this path. Toennies and his associates ('34) have proposed a 'stepwise' oxidation of cystine and cysteine and have been successful in the synthesis of some sub-oxidation products of cystine and cysteine. The formulae of the products of partial oxidation of cysteine are here presented.

$$\underset{\text{cysteine}}{RSH} \rightarrow RSOH \rightarrow RSO_2H \rightarrow \underset{\text{cysteic acid}}{RSO_3H}$$

While the exact significance of these compounds is yet to be demonstrated, preliminary results of their study biologically are promising (Hammett, '33).

Mention should also be made of existence of the thiocyanate ion in small amounts in blood, saliva and other secretions, and urine. Neither the significance nor the origin of this sulfur compound is known.

Of the reactions for the qualitative detection of the biologically important sulfur compounds, the nitroprusside-ammonia reaction has perhaps the greatest interest. This reaction of sulfhydryl compounds with sodium nitroprusside in the presence of ammonia to yield a purple-red complex, first described by Gola in 1902, was little used, until Arnold in 1907 and Heffter in 1908 called attention to its significance. Since glutathione is present in tissues mainly in the reduced or sulfhydryl form, this reaction has been employed extensively as an index of its distribution and also served as an

outside indicator in the iodimetric titration of glutathione in the Tunnicliffe method. In the interpretation of data obtained by the application of this reaction to tissues or fluids, it must be borne in mind that the presence of other sulfhydryl compounds, as cysteine or ergothioneine, has not been excluded. A modification of this reaction, the cyanide-nitroprusside test, has been described for the detection of cystine. Cystine is first reduced to cysteine by sodium cyanide and the color of the nitroprusside complex is obtained on the addition of the nitroprusside. It must be remembered that the cyanide-nitroprusside reaction will be positive in the presence of any sulfhydryl compound or of any compound which by reduction yields a sulfhydryl group.

TABLE 2

Chemical methods used for the differentiation and determination of the biologically important sulfur compounds

1. Nitroprusside—ammonia reaction. SULFHYDRYL.
2. Cyanide—nitroprusside reaction. DISULFIDE.
3. Non-specific cystine methods. (These determine any SH group, free or formed by reduction of SS groups.)
   a. Colorimetric. Folin methods (reduction of phosphotungstic complex).
   b. Iodimetric. Okuda (oxidation of SH by I_3).
   c. Gravimetric. Vickery-White (precipitation of Cu^+ complex of sulfhydryl and gravimetric determination of S).
4. Specific cystine methods.
   a. Naphthoquinone-sulfonic acid reaction. Sullivan.
5. Methionine.
   a. Determination of methyl groups removable by HI. Baernstein.

The older methods for the determination of cystine were gravimetric; the cystine was precipitated from a protein hydrolysate or biological fluid and either weighed or the value calculated from the determination of the sulfur content of the crude precipitate of cystine. Although cystine is insoluble in pure solutions at its isoelectric point, the solubility is influenced by the presence of other substances, both organic and inorganic, so that complete precipitation from a protein hydrolysate or from urine is difficult to secure.

Four methods or modifications of these have been most commonly used for the determination of cystine in recent years (table 2). Three of these are based upon the general

reactions of the sulfhydryl group obtained by reduction of cystine with cyanide, sulfite or a metal and hydrochloric acid. These reactions are not entirely specific for the presence of cystine. The fourth, the naphtho-quinone-sulfonic acid method of Sullivan, is the only one which seems to possess a high degree of specificity. Of the non-specific methods, the iodimetric titration procedure of Okuda gives results most nearly comparable to those obtained by the Sullivan method. The Folin-Marenzi determination, which has the advantage of simplicity, undoubtedly gives values which are somewhat high due to the reaction of substances other than cystine or cysteine with the reagent. The recently published method of Vickery and White has not been used extensively, so that it is difficult to judge its value.

For the determination of methionine, only one method, that of Baernstein ('32 a), is as yet available. The validity of the hypothesis, upon which this method is based, that methionine alone of the constituents of the protein molecule yields methyl iodide on treatment with hydroiodic acid, must be established. In view of the demonstrated interrelationship between cystine and methionine in metabolism, it is desirable to have available complete and accurate information concerning the quantitative relationships of these amino acids in proteins. A satisfactory method for the determination of methionine in tissues of blood, urine, and other body fluids is much needed.

When protein is ingested by a normal individual, its sulfur is eliminated in oxidized form by the kidney. The chief fraction of the total sulfur eliminated is the inorganic sulfate sulfur fraction, precipitable by barium chloride without previous acid hydrolysis of the urine. In addition, a small portion of the oxidized sulfur is combined in ester linkage with certain aromatic or similar cyclic substances, the so-called ethereal or conjugated sulfuric acids, sulfur which is not precipitated by barium chloride unless the ester linkage has been split by acid hydrolysis. In addition there are normally present, in small amounts, sulfur compounds of organic nature, usually grouped as the 'neutral' or organic sulfur of the urine

(Amann, '33). A high level of protein in the diet results in a marked increase in the excretion of oxidized sulfur, but not of the organic sulfur. This indicates a ready and complete oxidation of the sulfur-containing amino acids of the protein molecule. When the amino acids themselves are ingested, a similar ready oxidation to sulfuric acid occurs provided the amounts fed are not greatly in excess of the physiological limits. The relative constancy of the organic sulfur excretion and its independence of the level of general protein metabolism are best shown by Folin's classical experiments (table 3).

TABLE 3

The influence of the protein of the diet on the distribution of urinary sulfur in man (Folin, '05)

	High protein, gm.	*Low protein, gm.*
Total nitrogen	16.8	3.6
Total sulfur	1.456	0.304
Inorganic sulfate sulfur	1.308 (90%)	0.184 (60%)
Conjugated sulfate sulfur	0.076 (5%)	0.040 (13%)
Organic sulfur	0.072 (5%)	0.080 (26%)
N: S	11.5	11.8

It is assumed that, since the sulfur of cystine or methionine is so readily oxidized by the animal organism, any intermediary product of this metabolism will also be readily oxidized on administration. This method for the study of cystine or methionine derivatives has proven of much value. While ready oxidation of the sulfur does not necessarily prove a physiological function, failure of oxidation can certainly be considered to indicate lack of any important physiological relationship. This may be illustrated by a consideration of cysteic acid. Cysteic acid may be easily prepared from cystine by oxidation. It has been postulated that the physiological path of oxidation of cystine is through cysteic acid and its derivative, taurine. However, neither of these substances administered to the usual laboratory animals increases the excretion of oxidized sulfur, indicating a failure of complete oxidation of the sulfur of these compounds. This argues strongly against the formation of cysteic acid as a

normal intermediary product of cystine metabolism. We have recently synthesized and studied in our laboratory peptides containing these two sulfonic acid derivatives (White, J., '33). It seemed possible that, if cysteic acid were formed in the oxidation of cystine, the preliminary oxidation might occur while the cystine was still in peptide form and that the sulfur of peptides containing taurine or cysteic acid might be oxidized. A parallel is found in the failure of oxidation of the pyrimidine ring when the free pyrimidine bases are fed, and the ready oxidation of the same bases when they are administered in a combination existing in the tissues, that is, as nucleosides, nucleotides, or nucleic acids. In the case of the sulfonic acid peptides, Mrs. White has been unable to secure evidence of any similar relationship; no oxidation of the sulfur could be demonstrated.

Attempts have been made to approach the problem of the reactions involved in the oxidation of cystine by 'blocking' certain reactive groups of the molecule and investigating the biological behavior of these derivatives. When one hydrogen of the α-amino group of an amino acid is replaced by an acyl or similar group, the resulting compound is not readily deaminized. It is usually believed that deamination is a primary step in the catabolism of amino acids. When normal deamination of cystine is prevented by 'blocking' the amino group of cystine by combination with a phenylureido or benzoyl group, the oxidation of the sulfur does not occur normally and the substituted derivative is excreted, in large part unchanged in the urine. In view of the fact that in the chemical laboratory, the sulfur of cystine is readily oxidized without affecting the α-amino group, it is difficult to explain this failure of oxidation in vivo when the α-amino group is 'blocked.' Similar experiments with S-substituted derivatives of cysteine, as S-benzyl- or S-ethylcysteine, have indicated that a second condition for ready oxidation of the sulfur is a free sulfhydryl group. It appears that for the oxidation of the cystine the most effective point of attack is the primary amine group and the least effective, the carboxyl, with the

disulfide or sulfhydryl group occupying an intermediate position. Despite much research, we still lack experimental data, adequate to explain the mechanism of oxidation of the sulfur of cystine. Whether study of the recently synthesized 'suboxidized' derivatives of cystine and cysteine of Toennies and his co-workers ('34) will afford the necessary evidence remains to be determined.

It is possible that these studies of the oxidation of the sulfur of cystine may throw some light on the mechanism of the synthesis of taurocholic acid of the bile. Garrod has estimated that 30 per cent of the total cystine metabolized is concerned in the synthesis of taurine. It is difficult to understand how accurate data for such an estimate can be secured in view of the lack of adequate information as to the volume of bile normally secreted and the paucity of satisfactory analyses of bile. It seems probable to the writer that in the synthesis of taurocholic acid, the cholic acid is first conjugated with cystine or some as yet unidentified intermediary product and that the product of conjugation is then further oxidized to taurocholic acid. If this hypothesis is correct, free taurine would not occur in the higher organisms and its formation from cystine would result from a deviation of the normal metabolic path. The facts that the amino group is not free in taurocholic acid and that cystine is not oxidized normally when the amino group is 'blocked' suggest strongly that the preliminary 'blocking' of the amino group of cystine by cholic acid results in a failure of the usual oxidative mechanism and leads to oxidation of the sulfhydryl to a sulfonic acid derivative. It must be admitted that experimental proof of this hypothesis is as yet lacking; on the other hand, no satisfactory evidence of direct synthesis from cysteic acid or taurine and cholic acid is available. It must be remembered, however, that free taurine is found in the tissues of certain invertebrates, notably the abalone.

The necessity of the free amino group for the normal metabolism of the sulfur of cystine finds a parallel in the metabolism of certain heterotrophic bacteria (Tarr, '34).

Hydrogen sulfide formation from cysteine by Proteus vulgaris is dependent upon the presence of a free amino group. Thus hydrogen sulfide is readily formed from cysteine, less readily from homocystine, but not from the product of reductive deamination of cysteine, β-thiolpropionic acid.

Valuable information concerning the metabolic interrelationships of the sulfur-containing amino acids may be obtained from growth experiments carried out with white rats on a diet deficient in cystine. In their classical experiments, Osborne and Mendel demonstrated that cystine was the first factor limiting growth in rats on a low protein diet, deficient in cystine, and that without altering the level of protein, normal growth could be obtained by the addition of cystine to the diet. These observations form the basis of many important investigations. If some other compound containing sulfur, when added as a supplement to the cystine-deficient diet, leads to an acceleration in the rate of growth, the assumption is made that the compound in question is interchangeable with cystine nutritionally or that it is a product of the normal catabolic reaction of cystine which may or may not be reversible. Thus neither elementary sulfur nor inorganic sulfates can serve as effective supplements to a cystine-deficient diet, indicating the necessity of sulfur in organic combination for the requirements of the higher forms. Cysteine supplements a cystine-deficient diet as effectively as cystine (Mitchell, '31), a finding in harmony with the usually accepted belief that the reaction cystine $\rightleftarrows$ cysteine is easily reversible in vivo as well as in vitro. Neither taurine nor cysteic acid, oxidation products of cystine, can replace cystine in the diet. Of particular interest also is the failure of the deamination product of cystine, the disulfide of α-hydroxy β-thiolpropionic acid, to replace cystine. In summary, it may be stated that with the possible exception of the decarboxylation product of cystine, cystinamine, concerning which the data are conflicting (Sullivan, Hess and Sebell, '31; Block and Jackson, '32), no derivative of cystine is known to replace cystine nutritionally in experiments with rats.

In view of the many negative results obtained with cystine derivatives, it is particularly significant that methionine has been observed to supplement a cystine-deficient diet as effectively as cystine (Jackson and Block, '32). This finding explains the peculiar results of Sherman and Woods ('25), who in biological assays of casein state that casein biologically was equivalent to a content of 1.3 to 2.5 per cent of "cystine (or cystine plus nutritionally equivalent sulfur containing radicles)." This result was difficult to explain in view of the fact that chemical analyses of casein showed a content of cystine of approximately 0.3 per cent. Since it is now known that casein contains a relatively high per cent of methionine (3.25 to 3.53 per cent) according to Baernstein ('32), it is possible to interpret the experiments of Sherman and Woods on the basis of the interchangeability of cystine and methionine. Methionine is the 'nutritionally equivalent sulfur-containing compound.' Sherman appears to have been the first to suggest the existence of a substance in proteins which functions similarly to cystine in nutrition. Although methionine had been isolated at the time of Sherman's studies, neither its chemical structure nor its wide-spread distribution in protein was known. Methionine has also been shown to influence the detoxication and conjugation of bromobenzene and the synthesis of the mercapturic acids in both dogs (White and Lewis, '32) and white rats (White and Jackson, '33) in a manner similar to cystine. Further evidence of interrelationship of cystine and methionine is to be obtained from studies of cystinuria to be discussed later.

The demonstration of the similar functions of cystine and methionine led to a more careful study of the metabolism of the more recently discovered of the two sulfur-containing amino acids. The sulfur of methionine, like that of cystine, is readily and completely oxidized to sulfates when methionine is administered to man or the common laboratory animals (Virtue and Lewis, '34; du Vigneaud, Loring and Craft, '34). It also appears that, when deamination is prevented by 'blocking' the amino group, the N-substituted derivatives of methionine, as well as those of cystine, are not

oxidized normally, although the evidence concerning this point is meagre (Virtue and Lewis, '34).

In experiments with rats and rabbits, the administration of methionine has been followed by the excretion of a substance which gives the reactions of a compound containing the disulfide linkage (SS) but fails to give the more highly specific Sullivan test for cystine (Chase and Lewis, '33; Virtue and Lewis, '34). In view of the fact that demethylation is not an uncommon biological reaction, and that demethylation of methionine has been demonstrated to occur readily in vitro by Butz and du Vigneaud ('33), the presence of the demethylation product of methionine, homocysteine, or its disulfide, homocystine, in the urine is suggested. Further support of the theory of demethylation as a reaction in the intermediary metabolism of methionine is afforded by the recent observations of Pirie ('34) that when slices of liver tissue of rats and methionine solutions are incubated together, the fluid gives, in all cases, a faint but definite nitroprusside reaction. The isolation of homocystine from the urine or other biological fluids is necessary to furnish the final proof of this hypothesis, at present supported by indirect evidence only.

The problem has been further clarified by the recent studies of homocystine as a supplement to a cystine-deficient diet (du Vigneaud, Dyer and Harmon, '33). The promotion of growth of young white rats under these conditions is as effective as when methionine or cystine is added as a supplement. Thus we are able to state with some certainty that these three amino acids are equivalent nutritionally, so far as growth is concerned.

It might be expected that demethylation is the normal path of metabolism of all the homologues of methionine. The sulfur of methionine, homocystine, and S-methylcysteine is oxidized readily in the organism of the rabbit (du Vigneaud, Loring, and Craft, '34). Demethylation does not appear to be the primary reaction of the catabolism of S-methylcysteine, however, since this compound is not an effective supplement of a cystine-deficient diet (du Vigneaud, Loring,

and Craft, '34). If demethylation were to occur prior to other changes in the molecule, cysteine or cystine should result and S-methylcysteine would be expected to serve as a source of cystine for growth. If the role of methionine as a substitute for cystine in growth experiments is to be explained by its conversion to homocysteine, the negative results with S-methylcysteine would indicate that the reactions of metabolism of the members of the homologous series of methylthiol amino acid are not similar.

Neither homomethionine nor the oxidized form of its demethylation product, pentocystine, function as do cystine, homocystine and methionine in promoting growth in rats on a diet low in its content of cystine (Dyer and du Vigneaud, '35). This indicates that the peculiar nutritional role of the three sulfur-containing amino acids which appear to be interchangeable in growth promotion is not simply a function of the disulfide (SS) or sulfhydryl (SH) group alone, but is related to the structure of the molecule as a whole. Only the sulfur homologues containing an α-amino group and three or four carbon atoms appear to possess significant biological properties.

Further evidence in support of the biological relationship of cystine and methionine is to be obtained from recent studies of the interesting 'error' of metabolism, cystinuria. For many years it has been known that the cystinuric individual, while excreting considerable quantities of cystine particularly when on a high protein diet, was able to oxidize completely the amino acid itself, when fed. No satisfactory explanation of this anomaly was presented. Brand and coworkers ('35) have shown that the explanation presumably is to be found in the fact that the methionine and not the cystine of the diet is the source of the cystine excreted in cystinuria. Administration of cystine to a cystinuric failed to increase the cystine excretion while administration of methionine led to a marked increase in cystine. We have been able to confirm these findings with a cystinuric patient in our own laboratory. Brand has also observed that cysteine augments the

cystine excretion. He postulates a direct chemical conversion of methionine to cysteine, which is difficult to picture in the light of any known mechanism of chemical transformation in the organism, and a path of metabolism of cysteine distinct from that of cystine. Homocystine was oxidized readily and completely by the cystinuric, while the administration of homocysteine led to a significant augmention of the cystine content of the urine.

Despite the many advances in recent years, the main problem of the function of cystine remains unanswered. What is the function of the sulfur? Is the equilibrium cysteine $\rightleftarrows$ cystine as easily reversible in vivo as is usually assumed? Is the function of cystine related to the SS or SH linkage? The fact that homocystine is equivalent to cystine in nutrition might suggest this. On the other hand, it will be recalled that the α-hydroxy-derivative of cystine, the so-called 'desaminocystine,' which differs from cystine only in the absence of the α-amino group, cannot replace cystine, a fact which speaks against the function of the SS group alone.

Are we to postulate an actual synthesis of cystine from methionine through homocystine, or homocysteine, or is there an intermediary product common to both these amino acids through which they function in metabolism? When methionine is fed to a dog receiving bromobenzene, is there synthesized a new, as yet unknown, conjugation product, a mercapturic acid of which acetylhomocysteine is a constituent rather than the usual acetylcysteine, or does the methionine when administered take over some necessary metabolic function of the cystine, thereby making available cystine for mercapturic acid synthesis? If demethylation is the primary reaction of the intermediary metabolism of methionine, is the methyl group liberated made available for purposes of synthesis, as for example, the methylation of glycine as a stage in the synthesis of creatine? What is the relation of cystine or methionine to insulin? To glutathione? What is the function of the latter? These are some of the problems of sulfur metabolism, the solution of which is essential before

a coherent picture of the relation of the sulfur compounds to each other and to the function of the living organism can be presented.

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